



Consommation
et Corporations Canada

Consumer and
Corporate Affairs Canada

Bureau des brevets

Patent Office

Ottawa, Canada
K1A 0G9

(21) (A1)

2,029,906

(22)

1990/03/19

(43)

1990/09/21

(52)

C.R. CL. 195-1.31
195-1.38
195-128.1
167-135

(51) INTL.CL. ⁵ C12P-021/00; C12N-001/21; A61K-039/02

(19) (CA) APPLICATION FOR CANADIAN PATENT (12)

(54) Vaccines for Combatting Septicemic Bacteria

(72) Audonnet, Jean-Christophe - France ;
Bruneau, Patrick - France ;

(73) Rhone Merieux Société Anonyme - France ;

(30) (FR) 89/03626 1989/03/20

(57) 16 Claims

Notice: The specification contained herein as filed

Canada

2629000

(57) Abstract

The bacteria expressing iron-regulated outer membrane proteins of which certain are siderophore or transferrin receptors can be used in a vaccinal preparation. Said bacteria are obtained from a culture in a medium in which the iron content is reduced to a level for obtaining an increased expression of said proteins, and in particular of receptors, sufficient to induce, when said bacteria are used in a vaccine, the formation of antibodies preventing the specific recognition of siderophores by their receptors.

VACCINE AGAINST SEPTICEMIC BACTERIA, SEPTICEMIC
BACTERIA ANTIGEN PREPARATIONS, NEW BACTERIA AND
VECTORS FOR THE PREPARATION OF THESE ANTIGENS OR VACCINES

5 This invention relates to bacteria belonging to
genera including pathogenic bacteria and expressing
large amounts of external membrane antigenic proteins
regulated by iron.

It also relates to processes for the
production of these bacteria.

10 It furthermore relates to vaccines
against septicemic bacteria containing as an active
principle bacteria which express in large amounts
external membrane antigenic proteins regulated
by iron, or fragments of these bacteria or
15 antigens expressed by these bacteria.

It furthermore relates to other bacterial,
viral or other vectors allowing the expression
of these antigenic proteins, as well as to vaccines
containing these proteins as their active principle.

20 It furthermore relates to recombinant
live vaccines, notably bacterial or viral vaccines

expressing these antigenic proteins in the vaccinated organism.

It is known that apart from some lactobacilli iron is a necessary nutriment for all living forms including bacteria. These need iron to be able to multiply in a host cell. The capacity of the bacterium to multiply in vivo is an essential factor of its virulence.

Although iron is present in large amounts in a human body, the bacterium only has a very small amount of free iron at its disposal in order to multiply.

Indeed by far the greater part of an animal host iron is intracellular (in the form of ferritin, haemosiderin or heme) and therefore its access is difficult. The small amount of iron which is present in the body fluids only exists in the form of extremely stable complexes, which are principally made up of two iron chelating glycoproteins : transferrin in the plasma and lactoferrin in the secretions. The existence of these glycoproteins strongly, but in a reversible manner, linking the iron, is necessary in order to allow its use by the cells, preventing its precipitation in the form of ferric hydroxide.

The plasma contains iron complexes in the form of haptoglobin-heme, ceruloplasmine, ferritin, lactoferrin and transferrin.

The major part of the iron is transported by transferrins. Three major classes of transferrins may be recognized : seric transferrin, lactoferrin and ovotransferrin.

The transferrin captures about 95% of the iron in the plasma and its saturation rate is only about 35% in the healthy individual.

Lactoferrin has a very small iron saturation rate and keeps its chelating properties in a wide pH range ; its presence in all secretions of the organism, that is to say at the level of potential microbial invasion sites, imposes a wider restriction for iron in these places than elsewhere in the organism.

The complexation of iron to glycoproteins results in that only a very small concentration of free ferric iron (10^{-19} M) remains, this being quite insufficient to allow a normal growth of bacteria.

In order to acquire the iron they need to multiply in the host, bacteria have a number of means available.

It seems that some microorganisms may obtain their iron by a mechanism implying direct interaction between the bacterial cell surface and the protein linking the iron in the host. However, this direct acquisition mode only affects a very limited number of species. Most bacteria, pathogenous or not, react to the lack of availability of the iron in a host, ^{or} in some aerobic environments, by producing iron chelating compounds called siderophores.

Siderophores are made up by molecules having a small molecular weight forming specific complexes with a very high affinity for the ferric ion. Their biosynthesis is regulated by the iron and their function is to supply the bacterial cell with iron.

These siderophores possess an extremely high affinity for the ferric ion (their association constant is about 10^{30} M^{-1}) which allows them to

displace the iron associated with the host protein or to solubilize the ferric iron which is precipitated in the form of hydroxide.

Most of previously identified siderophores belong to two chemical classes : phenolates-catecholates (deriving from 2,3-dihydroxybenzoic acid) and hydroxamates (deriving from hydroxamic acid).

The better known among siderophores belonging to the class of phenolates is the enterobactin which is excreted by the bacteria belonging to the genera Escherichia, Klebsiella, Salmonella and Shigella. This enterobactin is made up of a cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine and is the chemical compound having the highest known affinity with the ferric ion ($K_a = 10^{52} M^{-1}$).

Several enteric species synthesise another hydroxamate siderophore, aerobactin. This siderophore is particularly synthesised by septicemic or invasive Escherichia coli strains having a type Col V plasmid, or by Salmonella typhimurium and Shigella.

This biosynthesis of siderophores by bacteria is associated with the production of proteins at the outer membrane, some of these proteins behaving as receptors for siderophores, as well as mechanisms allowing iron transportation and release inside the bacterium.

The common characteristic of these proteins which are formed in the outer membrane, and are often called "IROMP" meaning Iron Regulated Outer Membrane Protein, is a size between 70 kDa and 90 kDa, and their synthesis as well in vitro

in a restricted iron environment as in vivo during infection.

The outer membrane proteins, or siderophore receptors, are therefore the second element of systems characterized as having a high affinity for the bacterial intake of iron (the first element being made up by siderophores).

Apart from these high affinity systems, many bacteria possess low affinity transportation systems which allow them to use ferric hydroxide in polymerized forms.

The absorption mechanisms for iron have been particularly studied with Escherichia coli which is the best genetically known micro-organism.

The high affinity iron transportation endogenous system in E. coli uses the siderophore, enterobactin. Enterobactin is synthesised and excreted in the medium when E. coli is placed in a restricted iron environment. The ferric enterobactin complexes are then taken up by the outer membrane (81 kDa Fep A protein) and transported to the cytoplasm. When internalized the iron is freed by ferric enterobactin hydrolysis, then reduced to ferrous iron.

E. coli's enterobactin system comprises at least thirteen genes. Seven genes (ent) are involved in the biosynthesis of the siderophore, and five genes (fep) code for transportation proteins.

Apart from the enterobactin system, E. coli septicemic strains excrete and carry an

hydroxamate siderophore, aerobactin.

It has been discovered in 1979 by P.H. WILLIAMS (37) that some Col V type plasmids carried genes for the aerobactin siderophore and its receptor located in the outer membrane and called Iut A protein (74 kDa protein).

Although the aerobactin has an association constant with the ferric ion which is lower than that of enterobactin, it however has structural properties increasing its capacity to take up the iron linked to the transferrin or to the lactoferrin.

After this was demonstrated in 1979 by P.H. WILLIAMS, many studies have shown that the aerobactin's iron transportation system played a major part in the virulence of pathogenic strains of E. coli and many other bacteria (GRIFFITH and al. (13)).

The presence of the aerobactin siderophore strongly favours the virulence of pathogenic strains.

Although aerobactin is less powerful than enterobactin as a chelating agent, it is active in much more varied environment conditions (enterobactin is very sensitive to oxidation and pH variations). Aerobactin therefore confers a higher degree of adaptation to the bacterium.

Besides, aerobactin is a better bacterial growth stimulator, and it seems that it is much more quickly excreted than enterobactin, probably because of a preferential genetic induction when E. coli is grown in the presence of a chelating agent.

With the aerobactin operon, the bacterium acquires an extremely efficient iron transportation system with a minimum number of additional genes, or only four genes for the synthesis of aerobactin which is a small simple siderophore, and one gene which codes for the outer membrane receptor. Indeed the other genes necessary for the transportation of hydroxamates are inherently present in all Escherichia coli.

The expression of all genes coding for membrane proteins, siderophore receptors and corresponding siderophores is regulated by a single protein, Fur, which acts as a repressor when the iron is available in sufficient amounts. The central regulation is superimposed on an individual modulation which regulates the expression of each system according to the state of the environment.

Some authors have grown bacteria, so as to increase the expression of IROMPs in environment with an iron deficiency with the help of chemical chelators, such as α , α' -dipyridyl (A. BINDEREIF et al). The cloacin receptor of Col V-bearing Escherichia coli is part of the Fe 3+ aerobactin system, J. Bacteriol., 1982, 150, 1472-475 ; C. MAROLDA et al. : Flanking and internal regions of chromosomal genes mediating aerobactin iron uptake system in enteroinvasive Escherichia coli and Shigella flexneri, J. General Microbiology, 1987, 133, 2269-2278 ; A. BINDEREIF et al. : Cloning of the aerobactin-mediated iron assimilation system of plasmid col V, J. Bacteriol., 1983, 153, 1111-1113 ; De LORENZO et al. : Aerobactin biosynthesis and transport genes of plasmid col V - K 30 in Escherichia coli K 12, J. Bacteriol. 1986,

165, 570-578 ; P. WARNER et al. : col V - plasmid-
 specified aerobactin synthesis by invasive strains
 of Escherichia coli, Infection and Immunity, 1981,
 33, 540-545). E. F. GRIFFITHS et al. have shown
 5 in : Synthesis of aerobactin and a 76000 Daltons
 iron-regulated outer membrane protein by Escherichia
coli K-12 - Shigella flexneri hybrids and by
 enteroinvasive strains of Escherichia coli, Infection
 and Immunity, 1985, 49, 67-71, that enteroinvasive
 10 strains of E. coli produce aerobactin and a 76
 K outer membrane protein when grown in a reduced
 iron environment in the presence of ovotransferrin.

The recently acquired knowledge on
 the iron absorption systems of bacteria has allowed
 15 one to explore new ways of fighting pathogenic
 bacteria.

It has been suggested to synthesize
 siderophoreanalogs which are toxic for the bacterium
 and may deceive the iron transportation systems
 20 in order to penetrate into the bacterial cell.
 But these synthetic chelators have a lower affinity
 for the iron (III) than natural siderophores, and
 they are unable to displace iron in transferrins.

ROGERS has suggested to form complexes
 25 between aerobactin and trivalent metal ions in
 order to use them as antimetabolites towards the
 enterobactin - Fe^{3+} natural complex. Only complexes
 formed with scandium (Sc^{3+}) and indium (In^{3+})
 have some antibacterial activity (ROGERS et al.
 30 (26) : ROGERS (27)).

It has also been suggested to adsorb
 phenolate type siderophores, which are aromatic

molecules, on some seric proteins, which then play the part of carrier molecules, this allowing the induction of specific antibodies against the siderophore.

5 Thus, BYERS (5) has described a vaccine against ^{the}phenolate siderophore which is produced by Aeromonas hydrophila (a bacterium which is responsible for human and fish septicemia), which has been assayed with fish. The siderophore is covalently
10 coupled with human or bovine albumine. Fish which are immunized with these preparations generate antibodies reacting against the siderophore. It is, however not specified if the antibodies which are formed are able to neutralize the siderophores.

15 One has also tried to prevent the bacteria from taking up siderophores with antibodies that would be specifically directed against the siderophore receptors.

20 BOLLIN et al. (4) report the results of a study indicating some passive immunization with antibodies against the outer membrane proteins regulated by iron, thus protecting turkeys from an Escherichia coli septicemia.

25 However, all efforts towards the elaboration of an efficient vaccine have, until now, failed because of the difficulties in having a bacterium express membrane proteins which are regulated by iron at a sufficient level in the bacterial culture.

30 This invention allows one to overcome this difficulty by suggesting the use in a vaccine as an active principle, of bacteria expressing in large amounts membrane proteins which are

regulated by iron and more particularly, siderophore receptors at a sufficient level to induce the development of antibodies which prevent the specific recognition of siderophores by their receptors.

5 An aim of the invention is to supply bacteria expressing outer membrane proteins regulated by iron (IROMPs), which may be used as protective antigens.

10 Another aim of the invention is to suggest the synthesis of outer membrane proteins regulated by iron of septicemic bacteria by genetic recombination.

15 Another aim of the invention is to supply large amounts of IROMPs, notably Iut A, and Fep A proteins, siderophore receptors, aerobactin and enterobactin in Escherichia coli and other families, through synthesis of these proteins by a genetic recombination.

20 Another aim of the invention is to supply vaccines containing as an active principle, bacteria or fragments of these bacteria having in their outer membrane large amounts of IROMPs and notably Iut A and Fep A proteins, as obtained by genetic recombination or by other processes.

25 Another aim of the invention is to supply vaccines containing as an active principle IROMPs, for example, Iut A and/or Fep A proteins or antigenic preparations incorporating these proteins.

30 This invention therefore uses bacteria expressing outer membrane proteins regulated by iron and some of which are siderophore receptors, which may be used in a vaccine preparation.

Bacteria according to the invention are characterized in that they express larger amounts of these outer membrane proteins, and more particularly, transferrin receptors, notably siderophore receptors, to induce, when these proteins are used in vaccine, the generation of antibodies preventing the specific recognition function by the receptor and thus putting an end to the iron supply of the pathogenic bacterium.

Bacteria which are used are preferably enterobacteria and they preferably excrete enterobactin and/or aerobactin siderophores.

Bacteria are preferably chosen among the group made up by Escherichia coli, Klebsiella, Salmonella, Shimurium, Shigella.

Bacteria preferably excrete together the aerobactin and enterobactin siderophores.

According to the invention and according to a first embodiment thereof, bacteria are obtained by growing naturally existing strains or strains that may be found in laboratories or collections in a minimal medium wherein the availability of iron is reduced to a level allowing a satisfying higher expression of membrane proteins. The culture is preferably grown in presence of a strong iron (III) chelating protein such as lactoferrins, these being chelators which advantageously establish an iron shortage with the same characteristics as those that are to be found in vivo.

Another object of the invention is also a process for producing such bacteria to be used for the preparation of vaccines, characterized in that

said bacteria are grown in a culture medium containing an iron (III) chelating protein such as transferrins, and notably lactoferrins.

5 As a minimum medium, one can use that which is described for instance by SIMON and TESSMAN (30).

10 However, this embodiment remains difficult to apply, because with the iron chelators which are generally used one must sufficiently reduce the iron content in the culture medium so as to obtain a sufficient expression of the outer membrane proteins. Often, small amounts of iron remain, preventing the expression of membrane proteins (iron from the fermentor, for instance 15 or from pipes which are, generally, made of stainless steel). One must then resort to comparatively complex processes in order to lower the iron content to a level allowing the expression of the external membrane proteins of bacteria, and their application 20 is costly.

According to a second embodiment of the invention, which is also the preferred embodiment, bacteria expressing in large amounts the outer membrane proteins, siderophore receptors, are 25 transformed by recombinant plasmids.

Indeed, the advantages of synthesizing the outer membrane proteins, siderophore^{receptors} or transferrin, through genetic recombination, are many :

- 30 - it allows one to generate an important expression of these proteins, whatever the iron concentration in the culture medium,
- it allows one to study immune reactions directly aiming at these proteins, while excluding

any other constituent of the original strain,

- it represents the cheaper solution for the expression of membrane proteins in an environment wherein iron is always present (fermentors, pipes and sundry stainless steel equipment).

If the applicant more particularly aims at the synthesis of Iut A and Fep A proteins, aerobactin and enterobactin siderophore receptors of E. Coli, it can be understood that the below described genetic recombination methods will apply by analogy with the synthesis of membrane proteins (IROMPs), siderophore receptors, aerobactin and enterobactin or transferrins from pathogenic bacteria other than E. coli.

The invention therefore also relates to the preparation of E. coli Iut A and/or Fep A proteins by genetic recombination.

The Iut A protein may be synthesized by a process according to which, in particular :

- one isolates the plasmid or chromosome from Salmonella, Shigella or Klebsiella pathogenic E. coli strains, bearing the aerobactin operon,
- one separates from the plasmid or chromosome a fragment containing the iut A gene,
- one links said fragments with a cloning vector,
- one inserts the clones having integrated the iut A gene in an expression vector (for example GTI 001 plasmid),
- one then expresses the Iut A protein by growing the clones.

The Fep A protein is obtained :

- by isolating from a plasmid (for example pMS 101 plasmid built by LAIRD and YOUNG (19)) or from a bacterial chromosome, E. coli, Salmonellae or Klebsiellae, a fragment bearing the fep A gene,

- by cloning said fragment in a cloning vector,

- by inserting the fep A gene in an expression vector, preferably in a vector which is used for the expression of Iut A protein (GTI 001 plasmid),

- by the expression of the Fep A protein by growing the clones.

The expression vectors for Iut A and/or Fep A proteins may be bacteria and one prefers to use E. coli whose expression systems are best known. One may however also use other vectors, notably viral vectors or vectors made up of yeast, and which can be built by specialists.

Bacterial clones expressing Iut A and/or Fep A proteins may be multiplied in an appropriate medium at a sufficiently low temperature so as to prevent or limit the expression, generally below 32°C. The expression is then induced by rising the temperature, for example to 42°C during about 4 hours, so as to induce the expression of iut A and fep A genes.

One therefore obtains bacteria integrating Iut A and Fep A proteins as well as their proIut A and proFep A precursors, these having the shape of large size cytoplasmic inclusions.

These bacteria as used in a vaccine, as an active principle, induce the generation of antibodies directed against Iut A and Fep A proteins, preventing recognition by these proteins of their respective siderophores aerobactin and enterobactin, this therefore strongly reducing the iron supply to the bacterium and blocking its multiplication.

The invention therefore also relates to vaccines containing as an active principle recombinant bacteria expressing external membrane proteins regulated by iron.

The invention more particularly relates to vaccines containing as an active principle : recombinant bacteria or fragments of these bacteria, notably fragments of membranes integrating Iut A and/or Fep A proteins or their precursors, proIut A and/or proFep A ; or again Iut A and/or Fep A proteins and/or their precursors, for example, extracted from cytoplasm or extracted from the outer membrane of recombinant bacteria.

In another embodiment, the invention relates to vaccines containing as an active principle : bacteria which are homologous to septicemic bacteria, or fragments of these bacteria, grown in a medium with restricted iron supply, and which integrate in larger amounts Iut A and/or Fep A proteins and/or their precursors; / Iut A and/or Fep A proteins (and/or their precursors) being suitably extracted.

The latter vaccines are preferably prepared from bacteria which are grown in a medium containing a strong protein type iron (III) chelator,

notably transferrin, lactoferrin or ovotransferrin.

The invention would be better understood on reading the following specification, referring to the appended drawings, wherein :

5 Figure 1 is the protein profile as obtained by electrophoresis of a clone expressing Iut A protein,

 Figure 2 is a protein profile as obtained by electrophoresis of a clone expressing Pep
10 A protein.

 The abbreviations used in the following specification have the following meanings :

	Amp ^r	ampicillin resistant
	Clo ^s	cloacin sensitive
15	dATP	deoxy-adenosin-triphosphate
	EDTA	ethylene-diamine-tetraacetic acid.
	Ent	enterobactin
	E.O.P.S.	without specific pathogenic organisms
	IPTG	isopropyl-β-thiogalactopyranosid
20	kpb	bases kilopair
	LB	luria broth
	OMP	Outer-membrane protein
	PAGE	polyacrylamide gel electrophoresis
	pb	bases pair
25	PBS	phosphate buffered saline
	SDS	Sodium dodecyl sulfate
	ST	Simon and Tessman
	TEMED	N,N,N',N'-tetramethylene diamine
	Tris	tris-hydroxy-aminomethyl-methane
30	tet ^r	tetracyclin resistant.

MATERIALS AND METHODS

I. MATERIALS

1. Strains

Table I recapitulates the various strains.

5 The pathogenic strains used are septicemic strains from calves or chicks and are to be found in the RHONE-MERIEUX strain collection.

10 The host strains . . . used for cloning, sequencing and expression are all derived from Escherichia coli K 12.

These strains may be easily replaced by other wild septicemic strains or laboratory strains;

2. Plasmids

15 Origin and characteristics of plasmids used for cloning and expression are presented in Table II.

3. Media

SIMON and TESSMAN minimum medium (30)

20 Contents :

	NaCl	5.8	g	
	KCl	3.7	g	
	CaCl ₂ , 2H ₂ O	0.15	g	
	MgCl ₂ , 6H ₂ O	0.10	g	
25	NH ₄ Cl	1.10	g	
	Na ₂ SO ₄	0.142	g	
	KH ₂ PO ₄	0.272	g	
	Tris	11.20	g	
	H ₂ O	1000	ml	pH 7.4
30	gsp			

The only carbon source is sodium succinate added to a final concentration of 10 g/l.

In order to establish a limitation to the iron in this medium, one adds ovotransferrin (SIGMA) at a final concentration of 250 $\mu\text{g/ml}$ (One may have concentrations above 500 $\mu\text{g/ml}$).

5 The iron-rich control medium is obtained by adding $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ (MERCK) at a final concentration of 40 μM .

M9 MANIATIS minimum medium (29)

	Na_2HPO_4	6.0 g	
10	KH_2PO_4	3.0 g	
	NaCl	0.5 g	
	NH_4Cl	1.0 g	
	H_2O qsp	1000 ml	pH 7.4

to this basic medium are added :

15	Mg SO_4 1M	2 ml/1000 ml
	glucose 20 %	10 ml/1000 ml
	CaCl_2 1M	0.1 ml/1000 ml

- LB rich medium (MANIATIS and al. (23))

	bactotryptone	10 g	
20	Yeast extract	5 g	
	NaCl	5 g	
	H_2O q.s.p.	1000 ml	pH 7.4

BTS rich medium (BIO MERIEUX)

	Biotryptase	17 g	
25	Biosoyase	3 g	
	NaCl	5 g	
	K_2HPO_4	2.5 g	
	glucose	2.5 g	
	H_2O qsp	1000 ml	pH 7.3

- BHI rich medium (heart-brain BIO MERIEUX)

30	Calf brain infusion	200 g
	ox heart infusion	250 g

Bio-gelytone	10	g
NaCl	5	g
Na ₂ HPO ₄	2.5	g
glucose	2.0	g
H ₂ O	1000	ml
usp		pH 7.4

- "M9 SP" rich medium for the expression medium :

SP (tryptone 3.2% : yeast 2%) 100 ml

M9 (6.6 x concentrate filtered on 0.22 μ m

filter (Millipore) 15 ml

5 MgSO₄ 100 mM 1.5 ml

FeCl₃ 0.1 mM 1.5 ml

vitamin B1 (5% solution) 1.5 ml

The solid media have the same composition as that of the corresponding liquid media and contain 12 g agar per liter of the medium.

10

Antibiotics are used in solid and liquid media at the following final concentrations :

Ampicillin 25 μ g/ml

tetracycline 12.5 μ g/ml

15 isopropyl- β -D-thiogalactopyranoside (IPTG) is optionally added at a final concentration from 0.05 mM to 0.4 mM.

Sterilization of liquid and solid media is made by autoclave at 120°C during 20 minutes.

20 Antibiotics, vitamin B1, sodium succinate

M9 6.6X, MgSO₄, FeCl₃, IPTG and ovotransferrin solutions are made in the form of concentrated stock solutions, and sterilized by filtration on a filter having 0.22 μ m porosity (Millipore).

25 After sterilization, the growth media are kept at room temperature.

Antibiotics, IPTG and ovotransferrin

solutions are kept at -20°C .

Other solutions are kept at $+4^{\circ}\text{C}$

II. METHODS

1. Bacterial cultures

5 Apart from clones with structures built
in the pGTI 001 expression vector, which are grown
at $+30^{\circ}\text{C}$, all cultures are made at $+37^{\circ}\text{C}$, while
stirring, during 18 hours.

10 Whenever necessary, bacterial growth
is estimated by measuring the suspension turbidity
at 600 nm, with the help of a BECKMAN DU 40 spectro-
photometer.

15 Cultures are usually made in a volume
of 2 ml after seeding with a colony. Cultures
in a more important volume (20 ml to 1000 ml)
are made by seeding to the 1/100th with a preculture
in stationary phase.

2. Sensitivity toward bacterocins

20 Productions of cloacin DF 13 and colicin
B are made with Enterobacter cloacae DF 13 and
Escherichia coli 1300 strains, respectively, according
to the process described by DE GRAAF (DE GRAAF
et al. (8) and (9)).

25 These strains are grown at $+37^{\circ}\text{C}$, in
a BHI medium, until the optical density reaches
0.5 (1 cm, 600 nm). Mitomycin C is then added to
the growth medium, so as to reach a final concentration
of 1 $\mu\text{g/ml}$, which allows one to induce the synthesis
of bacteriocins. This culture is prolonged during
30 6 hours, at $+37^{\circ}\text{C}$, until lysis phase. Bacterial
bodies are centrifugated (8000 g, 30 nm, $+4^{\circ}\text{C}$)
and the supernatant is harvested. Ammonium sulfate
is then slowly added at $+4^{\circ}\text{C}$, until the concentration

reaches 365 g/litre.

The supernatant is taken up in 0.05 M phosphate buffered pH 7.0 and dialysed against several succeeding baths of this buffer. The dialysate
5 is filtrated over 0.22 μ m (Millipore) and kept at -20°C.

About 10^9 bacteria of the clone under study are spread on LB agarose containing the appropriate selection antibiotic. When the deposition
10 liquid is completely absorbed, one places at the center of the Petri dish 75 μ l of the bacteriocin solution. When this drop has itself dried, the Petri dish is placed in an incubator (+30°C or +37°C, as the case may be) during 18 heures. Clones
15 which present a growth inhibition around this deposition have become bacteriocin-sensitive. Clones which resist the toxic effect of bacteriocin on the contrary exhibit a uniform bacterial mat.

3. Preparation of antisera directed against the outer membrane proteins regulated by iron

20

The protocol which is used is a repetition of that which is described by BOLIN and JENSEN (4).

The outer membrane proteins regulated
25 by iron are separated by polyacrylamide gel preparative electrophoresis with sodium dodecyl sulfate added.

When the gels are coloured, the strip containing the IROMP to be used for the immunization
30 is cut off, then comminuted with distilled water, by passing through several needles having smaller and smaller diameters.

This ground product is injected to E.O.P.S.

(29) : Triton X-100 2% ; $MgCl_2$ 10 mM ; Tris/HCl
50 mM pH 8.0.

Incubation is carried out during 30
minutes at room temperature, while shaking every
5 five minutes. During incubation the cytoplasmic
membrane proteins are preferentially solubilized
by Triton X-100. Outer membranes are collected
through a new ultracentrifugation (111,000 g,
60 minutes, +4°C). The obtained pellet is thrice
10 washed in distilled water, finally resuspended
in 1 ml distilled water and frozen at -20°C for
storage.

6. Proteins dosage

The membrane extract protein concentration
15 is measured by a colorimetric method derived from
that published by LOWRY et al (20).

. To 0.5 ml protein solution to be dosed
are added 2.5 ml of the following solution :

1% $CuSO_4$ solution 1 ml
20 2% sodium tartrate
solution 1 ml
2% sodium carbonate
solution in 0.1N NaOH q.s.p.

After incubating 10 minutes at room
25 temperature, 0.25 ml 50% Folin reactant (Merck) is
added.

Incubation is carried out for 30 minutes
at room temperature, and the optical density of
the blue color which has evolved is measured
30 at 779 nm.

Protein concentration of samples is
determined with a standard interval prepared with
bovine serum albumin .

The optical density is proportional to the protein concentration in an interval of 5 to 200 $\mu\text{m}/\text{ml}$.

5 7. Techniques for the analysis of the
outer membrane protein composition : polyacrylamide
gel electrophoresis under denaturing conditions

Polyacrylamide gels are prepared according to the characteristics described by LUGTENBERG et al. (21).

10 The alignment gel has the following composition :

acrylamide-bisacrylamide (30/0,8 p/p)
5% ; Tris/HCl 130 mM pH 6,8 ; SDS 3,5 mM ; ammonium
persulfate 44 mM TEMED 8 mM.

15 The separation gel has the same composition as the alignment gel, except for the acrylamide/bis-acrylamide concentration (8 or 10%) and the Tris/HCl buffer 380 mM pH 8.8 concentration.

20 The migration buffer used has the following composition :

glycine	14.4 g
Tris	3.0 g
SDS	1.0 g
H ₂ O	q.s.p. 1000 ml
	pH 8.3

25 Extracts to be analyzed or purified by electrophoresis are diluted in at least an equal volume of the following dissociation buffer :
Tris/HCl 100 mM pH 6.8 ; glycerol 20% ; SDS 70 mM,
β-mercapto-ethanol 100 mM ; bromophenol blue 75 μM .

30 The thus diluted extracts are heated to 100°C during 5 minutes.

In order to analyze the outer membrane protein composition 30 to 50 μg proteins are deposited in each well.

For preparative electrophoreses, as much as 2 mg protein are deposited in the sole preparative well.

Migrations are carried out at +14°C during 5 hours at 160 V or 10 hours at 60 V (Vertical gel LKB apparatus). In order to increase the resolution of the outer membrane proteins regulated by iron, some electrophoreses have been under a voltage of 100 V during 16 hours. At the end of the electrophoreses, proteins are fixated and coloured during 30 minutes at room temperature with 1.2 mM Coomassie blue in a (50:10:50 v/v/v) methanol/acetic acid/water mixture. The unfixed colour is eliminated with several succeeding (15:145 v/v/v) methanol/acetic acid/water baths at 4°C. Once decolorated, the gel is photographed and dried.

The outer membrane protein profiles for each strain may be analyzed by densitometry (LKB ULTROSAN laser densitometer).

20 8. Detection and analysis of anti-IROMPs antibodies

The presence of specific anti-IROMPs antibodies is investigated with the microplate ELISA technique (E. J. and PERLMANN (10) ; COULTON (7)). Antigens which are coupled to the solid phase are fractions which are very much enriched in proFep A protein.

The reactivity of anti-IROMPs antibodies is made with a rabbit anti IgG conjugate (or chicken peroxidase (Nordic). The substrate used is 4-aminophenylene-diamine. Readings are made at 492 nm.

- "Western-blotting" technique

Proteins which are separated by polyacrylamide gel electrophoresis with SDS are transferred on a polyvinylidene fluoride membrane (PVDF 0.45 μ m Millipore) according to the method described by TOWBIN et al. (34).

Transfer is made under 24 V during one hour with a BIOLYON apparatus using the following anodic and cathodic buffers :

10	anodic buffer	cathodic buffer
	Tris 0.3 g	Tris 0.3 g
	glycine 1.44 g	glycine 1.44 g
	methanol 100 ml	SDS 0.1 g
	H ₂ O qsp 500 ml	H ₂ O qsp 500 ml

15 After transfer, the PVDF membrane is saturated during one hour at +37°C in PBS buffer containing 1% skimmed milk.

The membrane is then cut into strips corresponding to electrophoresis tracks.

20 Sera to be studied are diluted into PBS buffer containing 1% skimmed milk, then contacted with membrane strips, 4ml diluted serum per strip.

After incubation for one hour at +37°C, while gently stirring, three 20 minutes washings are made in PBS buffer containing 2% skimmed milk at room temperature.

An anti IgG conjugate coupled to peroxidase, diluted to the 1/1000 in PBS containing 1% skimmed milk is added at a rate of 3 ml per strip.

30 After incubating one hour at +37°C while gently stirring, 3 20 minutes washings are made at room temperature in PBS buffer.

The diaminobenzidine substrate, diluted to 0.1% in a physiological water at pH 7.13 to which 30 volumes 0.1% H_2O_2 have been added, is then added to the whole. One then notices (after 5 to 20 minutes) brown coloured strips around proteins which are recognized by the antibodies in the serum under study.

The membrane is then washed in distilled water and dried up.

9. Plasmid DNA preparation method

Large plasmids contained in pathogenic strains are extracted according to the method published by KADO et al. (16).

Plasmids obtained during the various stages of cloning, undercloning, and construction in the expression vector are extracted according to the method of BIRNBOIM (BIRNBOIM and DOLY) (17). Plasmid DNA obtained after preparative extractions made with one of these methods is purified on cesium chloride gradient (MANIATIS et al. (23)).

When purified, the plasmids are taken up in Tris 10 mM ; EDTA 1 mM pH 8.0 buffer so as to reach a final concentration of 1 µg DNA/µl, and frozen at -20°C for storage.

10. DNA analysis and modification methods

All methods used for cloning, DNA digesting, restriction fragment analysis in agarose gel, modification of the ends of restriction fragments, hybridation with a radioactive probe after transfer of DNA on nitrocellulose membrane, are described by MANIATIS (MANIATIS et al. (23)). DNA has been sequenced and oligonucleotides have been synthesized according to special methods.

11. DN sequencing

Genes to be sequenced are subcloned in M13 mp 18 and M13 vectors (YANISCH-PEPPERON et al. (38)) and sequenced according to the chain end method by deoxynucleotides (SANGER et al. (29)). Labelling of the various chains is made with ^{25}S (AMERSHAM).

Sequencing proper is made with reactants and sequencing kit enzymes Amersham and Sequenase (USB). Electrophoreses in a polyacrylamide gel with urea are made in a Sequi-Gen (BioRad) apparatus.

Sequencing data are processed with Microgenie (Beckman) software.

12. Oligonucleotides synthesis

The various oligonucleotides which are necessary for the constructions ⁱⁿ the expressing vectors or to the mutageneses are synthesised according to the cyano-ethyl-phosphoramidites method on an Applied Systems 381-A apparatus.

These oligonucleotides are directly used after deprotection and precipitation with ethanol.

13. Directed mutagenesis method

Mutagenesis is made according to the method described by ECKSTEIN (TAYLOR et al. (33) : NAKAMAYE and ECKSTEIN (24)) with the directed mutagenesis kit sold by the Amersham Company.

RESULTS

1. ISOLATION AND ANALYSIS OF mut A

The source of the aerobactin operon has been searched on plasmids born by 15371, 15372 and 15373.

After purification on caesium chloride gradient, these plasmids are digested by Bam HI, Hind III, Pvu and Sal I restriction enzymes (Boehringer). The various restriction fragments of each plasmid are separated on 0.8% agarose gel and transferred on a nitrocellulose membrane according to the "Southern blot" (SOUTHERN (31)) method. These fragments are then hybridated with two radioactive probes (labelled with 32p by displacement of cut) prepared from pABN1 plasmid restriction fragments bearing the aerobactin operon of pCol V-K30, (BINDERBIF and NEILANDS (2)). It has been found that the gene coding for the IutA receptor exists on all plasmids on a 6.6 kb Bam HI-Bam HI restriction fragment. These results are a confirmation of the authors' work of undercloning this gene from an equivalent pCol V-K30 fragment (KRONE et al. (17)).

- Cloning of plasmid DNA fragments bearing the Iut A gene

Plasmids of 15393, 15972 et 16003 strains are digested by Bam HI restriction enzyme. After electrophoresis on 0.8% agarose gel, the gel strip containing the 6.6 kb fragment of each plasmid is cut and the DNA is electroeluted.

The three 6.6 kb Bam HI-Bam HI fragments are ligated separately and the pAT 153 vector digested by Bam HI. The three ligation mixtures are used to transform competent HB 101 bacteria (table 17).

The DNA of ampicillin-resistant, tetracycline sensitive clones is extracted (by the method of MIRANDA and JACOB) and digested by Bam HI in order to check the size of the insert.

Clones having integrated the 6.6 kb fragment are selected on the basis of their sensitivity to cloacine DF 13.

For each starting plasmid a cloacine sensitive clone is kept for analyses and further build ups. They are the following clones :

Strain 15393	clone HB 101 p 5 - 15
Strain 15972	clone HB 101 p P - 13
Strain 16003	clone HB 101 p 4 - 18

10 - Clones analysis

A study of the expression of *lut A* receptor.

Having been able to select the desired clones with a cloacine sensitivity test shows that there is an expression of the receptor to cloacine and aerobactin. Expression of the *lut A* gene born by the 6.6 kb Bam HI-Bam HI fragment probably depends from a weak promoter situated on this fragment (KRONE et al. (17)) and does not depend on the iron concentration as does the main promoter of the operon aerobactin. Indeed, the sensitivity to cloacine is demonstrated with a culture on iron-rich ampicillin-LB gelose.

In order to study the expression level of the *lut A* receptor, the various clones are grown in LB-ampicillin medium (for one night at 37°C) with or without ovotransferrin (500 µg/ml).

The outer membrane proteins of each of the three clones are extracted and analyzed on polyacrylamide gel with SDS.

Whatever the growth conditions, it is possible to see a 76 kDa supernumerary protein

expression in cloacine sensitive clones.

- Restriction Maps

Plasmid DNA of clones 4-18, 5-13 and P-13 is extracted in large amounts and purified on caesium chloride gradient.

Restriction maps showing the three 6.6 kb Bam HI-Bam HI fragments which have been cloned are drawn with Sgl II, Bst E II, Cla I, Eco RI, Kpn I, Pst I, Pvu II and Sma I.

These three maps are totally identical and correspond to the restriction map of the 6.6 kb Bam HI-Bam HI fragment of p ColV-K30 aerobactin operon deduced from maps published by BINDEREF and NEILANDS (1,2) and KRONE et al. (17).

These four maps are represented in table V.

- Sequencing of the A genes

When comparing restriction maps for the three 6.6 kb Bam HI-Bam HI fragments with the restriction map as deduced from the sequence of the int A gene (KRONE et al. (18)), it appears that the int A gene is entirely to be found on a 3.2 kb Bst E II-Bst E II restriction fragment (table VI).

This fragment is isolated by electrophoresis, religated upon itself with T4 phage ligase (Boehringer) and digested with several restriction enzyme systems. The various fragments thus obtained (size 150-600 pb) are isolated by GeneClean (Bio 101) and subcloned in the M13 mp 18 and mp 19 vectors previously digested with the appropriate enzymes. The sequence of each subclone is then

determined according to SANGER's method with the use of Amersham and Sequenase (USB) sequencing kits.

Only the 3.2 kb Bst E II-Bst E II fragment of clone P-13 has been entirely sequenced. The two remaining lut A genes have been sequenced between the Bgl II (1) site and the Eco RV (2396) site.

The Bst E II-Bgl II region of clone P-13 has been compared with the sequence of the luc D gene located just upstream of lut A (HERRERO et al. (14)) and the Pvu II-Bst E II region of this clone has been compared with the sequence of ^{of} IS1 element (OHTSUBO and OHTSUBO (25)).

These comparisons as well as the comparisons of the three lut A genes with the sequence of the lut A gene of pColV-K30 are presented in table VII.

Analysis of these four sequences reveals that the lut A gene was extremely well kept at the molecular level. Apart from one or two bases, the three lut A genes isolated from E. Coli strains of animal origins are identical. Differences observed with the sequence published by KRONE et al. (13) are minimal.

The three lut A gene under study are 99.77% homologous to the lut A gene of pColV-K30.

Regions where differences have been demonstrated are represented in table VIII. (Numbers relate to the position of bases in sequences presented in table VII).

Two important regions are totally preserved :
 the sequence coding for the signal peptide and
 the consensus sequence ("Ton B box") typical
 of the outer membrane protein receptors whose
 function depends from Ton B.

The existence of four inserts in relation
 to gene iut A of ColV-K30 has been demonstrated
 on each of the three sequenced genes. These inserts
 trigger limited changes in the reading frame.

Thus, the primary structure of iut A proteins
 coded by isolated plasmids of the strains under
 study is a little larger (+ 8 amino acids) than
 that of protein iut A of p ColV-K30. The sequence
 of isolated iut A genes of the strains under study
 codes for a 733 amino acid polypeptide comprising
 a 25 amino acids signal peptide which is identical
 to that of the iut A polypeptide of strain ColV-
 K30. The calculated mass of the mature protein
 is 78097 daltons, which differs slightly from
 the size observed on gel (76 kDa). However, the
 changes in the primary structure are not sufficiently
 important to alter the secondary structure and
 the hydrophilicity profile.

2. EXPRESSION OF CLONED IUT A AND fed A GENES

- Characteristics of the vector under study

The expression vector used is the
 GT1 001 plasmid built up by the Mérieux Institute
 genetics engineering laboratory.

The genes it bears and its restriction
 map are presented in table IX.

This plasmid may be built up as follows :

Plasmid pBRTac, made up by pBR322.

(Bolivar F. et al. Gene 2, 95-113 (1977)) propagating between HindIII and BamHI ^{the} promoter Tac (Ammann

3 D. et al., Gene 25, 167, (1983)), has its XhoI site destroyed by the Kleenow polymerase (also called "the kleenow") to give plasmid pBRTacX⁻.

This plasmid is digested by NcoI, treated by the kleenow, then digested by AwaI, and its smaller
10 fragment is ligated to the pMC9 fragment (Casadaban M.J. et al., Journal of Bacteriology, 143, 971-980 (1980)) digested by MstII, treated with the kleenow and then digested by AwaI bearing gene Lac i and the replication origin of pBR322.

15 The resulting plasmid (named pBRLacix⁻) is digested by HindIII, treated with the kleenow and then digested by PstI, and the 2350 bases pair (or "pb") fragment is ligated to the 2300 pb fragment of pBRTac digested by EcoRI, and then
20 treated with the kleenow, and digested by PstI, thus creating plasmid pBRTaci. The latter's 4406 pb fragment, obtained by digested with EcoRI and PstI is ligated with a 1686 pb fragment digested by EcoRI and PstI and derived from pBR322 sequences
25 and bearing the pBR322's tetracycline resistance gene.

The obtained plasmid is called pBRTaciTet.

The latest replication origin is separated by digestion with BamHI, and the remaining 2096
30 pb fragment is ligated to the 2033 pb fragment of pATT53 (Twigg A.J. & Sherratt, D. Nature, 293, 216-218 (1980)) digested by XhoI, thus creating plasmid pATTaciori.

This plasmid is digested by EcoRI, then treated with the klenow, and digested by Aval and the 2704 pb fragment is ligated with a fragment bearing promoter Pr and its thermosensitive CI857 repressor of the 3076 pb Lambda bacteriophage derived from pCQV2 (Queen C. et al., J. Mol. Appl. Genet. 2, 1-10 (1983)) by digestion by PstI, treatment with the Mung Bean nuclease, and partial digestion with Aval. The resulting plasmid is called pGTI001.

The replication origin of this plasmid is under control of promoter lac, which allows one to regulate the number of copies by growing the bacteria in a medium containing various IPTG (gene lac i inducer) concentration.

The gene to be expressed is placed under control of phage CI 857 strong "Pr" promoter (the phage's repressor being thermosensitive). The ATG of the gene to be expressed is replaced by the ATG of gene cro. This ATG is created by partial Bam HI digestion of p GTI 001 followed by digestion with Mung Bean nuclease so as to obtain a blunt end.

The gene to be expressed is inserted in phase (starting from codon number 2) between the blunt ATG end and the XhoI site. An ending signal for the transcription, placed just downstream from the XhoI site, avoids the production of too long messenger RNAs.

Other plasmids of this kind able to express gene lut A (or fep A), are easy to obtain or to build and one knows such plasmids wherein the gene to be expressed is controlled by a promoter whose repressor is thermosensitive.

- Construction of the expression vector of gene lut A

lut A genes isolated from strains 15972 and 16003 are cloned with their signal sequence at the level of the expression site Bam HI (899) of p GT1001.

To obtain a blunt 5' end, starting with the second amino acid of the signal sequence (this time methionine), a two strands synthetic oligonucleotide is used to replace the region comprised between the initiation ATG and the only Acc.I site situated in 5' of the coding sequence. The sequence of this oligonucleotide is presented in table X.

The two complementary strands of this oligonucleotide are synthesised and the double strands form is obtained by heating to 90°C in a NaCl 50 mM, Tris 10 mM, MgCl 10 mM, pH 7.5 buffer, of an equimolar mixture of the two single strands, followed by a slow cooling down to room temperature.

The strategy which is followed in order to insert gene lut A of strain 15972 (clone p P-13) is presented in table XI.

With a not very satisfying yield, a modified strategy is adopted to put in phase gene lut A of strain 16003. This new strategy uses a sub-cloning of the Eco RI-Eco RI region of the intermediary construct (step 4, table XII) in vector pSB 118. This vector is a pUC 18 derivative. It has a "polylinker" between two Eco RI sites. Sub-cloning of the Eco RI-Eco RI fragment in this vector has thus allowed one to put in phase gene lut A while avoiding partial digestions. (Table XII).

Restriction map of the

GTI P-2 (gene lut A of strain 16003) and GTI P-5 (gene lut A of strain 16003) expression plasmids thus obtained are presented in table XIII.

Clones obtained after renewed ligation with the double strand "lut" oligonucleotide are selected on the basis of the preservation of site Acc I in gene lut A and the disappearance of site Bam HI in p GTI 001. All clones presenting this restriction profile have been controlled at the expression of protein lut A.

- Control of the expression of lut A

Qualitative control

The selected clones are grown in M9 SP tetracycline medium with 0.4 mM IPTG at 32°C (start of induction). The sensitivity of these clones towards cloacine is examined by the above described method.

Two clones out of 25 are positive for the constructions GTI-lut 15972.

Three clones out of 6 are positive for the constructions GTI-lut 16003.

The cloacine sensitive clones are grown in 50 ml tetracyclin M9 SP medium containing 0.4 mM IPTG.

The culture is made at a temperature of 30°C till the optical density reaches 1. Induction of gene lutA expression is then made by continuing the culture for four hours at 42°C. The bacteria are centrifugated and their total proteins are analyzed by polyacrylamide gel-SDS electrophoresis. This allows one to directly appreciate the importance

of Iut A protein production (figure 1). The analysis of clone CMX 603 GTI P-2 reveals that protein Iut A and its precursor represent, after induction, 25% of the bacterium's total proteins. The sole Iut A protein represents 30% of the outer membrane proteins.

- Construction of the expression vector for gene *lep A*.

Characteristics of the initial clone
p MS 101

Following the results of CODERE and EARMHART (6) indicating that gene *lep a* is located on a 6.3 kb Bam HI-Bam HI fragment of plasmid pMS 101 constructed by LAIRD and YOUNG (19), this fragment is sub-cloned and vector pBR 322 digested by Bam HI. The restriction map of the obtained plasmid (F-1) appears similar to that of plasmid pITS 1 (FLEMING et al. (11) -table XIV).

The publication of the sequence of *lep A* (LUNDRIGAN and KADNER) (22) has enabled one to precisely locate this gene on the restriction fragment Ssp I-Spu I 2530 pb of plasmid F-1.

The following strategy is used to insert gene *lep A* into p GTI 001.

An oriented mutagenesis is made in terminal 5' region of the coding region in order to transform sequence :

5'ATGAACAAG 3'

into a HpaI restriction site

MET ASN LYS

--

G T T A A C A A G

This site is cut into blunt-ended ends in the following manner GTT AA C. This allows direct ligation of gene fep A with the ATG end created in p GTI 001.

The mutagenesis is made according to the ECKSTEIN method from ^{an} oligonucleotide (table XVI), after sub-cloning of 800 pb Ssp I-Eco RI fragment in the replicative form of phage λ 13 mp 19.

The mutated fragment is entirely sequenced in order to check that the sequence had not been modified elsewhere than at the desired site.

Details of integration of gene fep A are summarized in tables XVI and XVII.

The various clones obtained after ligating fragment HpaI-XhoI 2350 pb in plasmid GTI 001 are selected by the presence of a 1700 pb Eco RI-Eco RI fragment.

- Control of the expression of Fep A Qualitative control.

The ligation mixture between the 2350 pb Hpa I- Xho I fragment and plasmid GTI 001 is used to transform competent bacteria RWB 18. This strain being fep A, it is colicine B resistant.

Clones having the desired restriction map (table XVII) are tested upon their sensitivity to colicine B.

One clone (RWB 18 GTI F-12) appears sensitive to the action of colicine B.

Quantitative control.

The expression of protein Fep A and its precursor is analysed on polyacrylamide-SDS gel (figure 2). Clone CMK 603 GTI F-12 expresses

Pep A and its precursor in a very large amount (20% of total proteins).

Protein Pep A represents 32% of the outer membrane proteins.

Physiological and morphological study of clones expressing *lut A* and *Pep A*

Growth potential

The growth potential of obtained clones is tested at various growing temperatures in LB tetracycline IPTG medium.

When seeded as a layer on LB tetracycline IPTG agarose 0.1 mM, all clones form bacterial mats which are sensitive to bacteriocins when grown at temperatures between 30°C and 34°C.

Above 34°C, bacterial mats no longer form. This has also been observed in liquid LB medium.

Sensitivity to bacteriocins is also found for IPTG concentrations of only 0.1 mM and at a temperature of 30°C. Therefore, there exists a level of expression for genes *lut A* and *pep A*, in the absence of an induction of vector p GT1 001.

Morphological study.

The various clones undergo morphological changes following overexpression of *lut A* and *Pep A*.

Bacteria, when observed with a phase contrast optical microscope after induction at 42°C during 4 hours, show a notable elongation (up to 10 times the average length of a normal *Escherichia coli*). However, the more striking

characteristic is the presence of one to several intracytoplasmic inclusions inside each bacterial body.

Inclusions as observed with the optical microscope may be found again when one observes with an electronic microscope after negative coloring of bacterial sections grown at -42°C during 4 hours.

These inclusions are peripheral and adjacent to the inner face of the cytoplasmic membrane.

3. Immunological properties of proteins Iut A and Fep A

Immunogenicity of Iut A and Fep A

Iut A proteins extracted from outer membranes of strains E. coli 15022, 15193, 15003 and recombinant strain Escherichia coli CVM 601 GT1 P-2 are isolated by preparative polyacrylamide gels and injected to rabbits according to the above described protocol.

Changes in the titre of anti-Iut A antibodies^{are} secreted by each rabbit assessed with the ELISA method, taking as an antigen a "granules" (precipitated proIut A protein) solution extracted from a culture of the strain E. coli CVM 601 GT1 P-2.

The positive reference serum used is a rabbit anti-Iut A protein serum of E. coli 15193-212, supplied by B. ODEGA.

The same protocol is followed for proteins Fep A extracted from membranes of strains E. coli 15022 and E. coli BW2 15 GT1 P11.

In all cases the rabbits react to the injection of protein Iut A and Pep A by producing a high titer of antibodies specifically directed against these proteins.

- Antibody properties of proteins Iut A and Pep A

The specificity of the various obtained antibodies is studied towards several outer membranes preparations (Strains E. coli 15022, 15393, CMX 603 GT1 P-2, CMX 603 GT1 B-5 and RWB 18 GT1 P-12). This study is using the "Western blot" method.

The four anti-Iut A sera which are prepared, as well as the standard anti-Iut A colV-K30 sera, specifically recognize a 76 kDa protein in all outer membrane preparations from strains expressing the aerobactin system. Whenever the (wild or recombinant) Iut A protein is used for their induction, the antibodies of one serum specifically recognize the Iut A protein expressed by bird or E. coli proteins and the two Iut A proteins synthesized by the recombinant strains E. coli CMX 603 GT1 P-2 and CMX 603 GT1 B-5.

The precursor of the Iut A protein, protein proIut A, is also specifically recognized by all anti-Iut A sera. (Immunoblots made with purified "granules" generated by strains CMX 603 GT1 P-2 and CMX 603 GT1 B-5).

Protein Pep 2 is not recognized by anti-Iut A sera and conversely, anti-Pep A sera do not recognize Iut A proteins.

Cloning of genes lut A and fepA in expression vector GT1 001 allows one to generate large amounts of proteins Lut A and Pep A as well as their respective precursors. Proteins Lut A or Pep A and their precursors which are synthesized following the induction of the transcription by the growth of bacteria at 42°C, rapidly accumulate in the form of large cytoplasmic inclusions (granules) which are visible with a phase contrast optical microscope. Observation with the electronic microscope of section of induced bacteria reveals that these granules are closely joined to the inner face of cytoplasmic membrane.

One must observe the importance of the expression level of Lut A and Pep A precursors (an average of 25% of total proteins under non-optimized conditions). Mature proteins make up as much as 35% of the protein content of the outer membrane. This percentage may be considered as the upper limit for the integration of this type of protein in the outer membrane. As a comparison, proteins Lut A and Pep A expressed by the wild strains Escherichia coli 15022 represent together 30% of the outer membrane proteins. It thus appears that the total expression of proteins Lut A and Pep A, and their precursors, by recombinant strains according to the invention is much higher than their natural expression.

The characterization of a sensitivity towards cloxacine DF 13 in clones expressing the Lut A proteins, and of a sensitivity to colicine B in those which express protein Pep A shows that

the synthesis and integration of these proteins in the outer membrane take place normally.

The identity of proteins obtained by genetic recombination with wild proteins is also demonstrated by the recognition of these proteins by antibodies directed against natural Iut A and Fep A proteins. One will note that these antibodies also recognize, with the same specificity, precursors proIut A and proFep A into intracytoplasmic inclusions.

Antibodies induced by proteins Iut A and Fep A as obtained by genetic recombination specifically recognize in the same way proteins Iut A and Fep A as expressed by various strains of pathogenic Escherichia coli.

Overexpression of receptors Iut A and Fep A by cloning of their genes on an expression vector, allows one to obtain in an iron-rich medium external membrane proteins which are functionally and antigenically identical to proteins expressed by pathogenic bacteria during their in vivo multiplication.

The synthesis of proteins Iut A and Fep A through genetic recombination thus has many advantages :

- it allows one to obtain proteins Iut A and Fep A in very large amounts, while freeing from regulation by iron,

- the synthesised proteins are functionally and antigenically identical to the proteins as expressed by pathogenic bacteria in their in vivo multiplication and they induce the production of antibodies,

- when used as an active principle in a vaccine, they induce the production of antibodies

preventing the specific recognition by membrane proteins regulated by iron, of the siderophores, thus stopping the supply of iron and blocking their multiplication in a host ; thus they allow, one to prepare very useful vaccines to prevent or fight infections including septicemias.

Vaccines may also be prepared simply from inactivated recombinant clones or from membrane fragments obtained by lysis of recombinant clones followed by purification, according to usual methods for the preparation of vaccines based on surface or wall antigens.

IV. PREPARATION OF IROMPS BY GROWING IN IRON-RESTRICTED MEDIUM.

1) Strain : E. Coli 078 reference 15022 :
origine : chicken

2) culture :

- Medium :

SI medium + succinate (Simon E.H. and Tasman (1963) Proc. Natl. Acad. Sci. USA 50, 526-532)

. addition of lactoferrin (250 µg/ml) to obtain a medium with an iron deficiency,

. or addition of $\text{FeCl}_3 \cdot 5\text{H}_2\text{O}$ (40 µM) :
for an iron-rich medium.

- Culture

- passing 3 times the strain in a iron-rich medium, this being followed by an adaptating stage in a deficient medium before final culture in a deficient medium,

- simultaneously, one proceeds to grow the same strain in an iron-rich medium,

- the cultures are made at 37°C during 24 heures.

3) Analysis:

At end of growth, in each medium, one proceeds to the following operations :

- harvesting by centrifugation,
- collecting ^{the} pellet in 0.2 M Tris HCl Ph 8, and ultrasonication,
- centrifugation (5000 g 30 minutes)
- recentrifugation of supernatant (100 000 g, 1 hour),
- taking up of the pellet in Tris HCl (50 mM, pH 8), MgCl₂ (10 mM), EDTA 1 mM, Triton 100 (2%) and agitating during 20 minutes at 37°C,
- centrifugating one hour at 100 000 g then reextracting the pellet,
- washing the ^{pellet} in demineralised water,
- analysing the pellet in polyacrylamide gel (PAGE SDS) under denaturing conditions (mercaptoethanol, SDS).

4. Result :

- Membranes of bacteria grown with lactoferrin : presence of two proteins, in large amounts, having apparent molecular weights 80000 da (enterobactin receptor Fep A) and 76000 da (aerobactin receptor Iut A),
- membranes of bacteria grown in iron-rich medium : no 76000 and 80000 da band.

V - PREPARATION OF VACCINES

Preferably, the active principles according to the invention will be associated, in the vaccines, with a conventional antigenic preparation in known human or animal antibacterial vaccines.

and notably in the case when one uses purified proteins.

These vaccines may show the active inventive principles in usual liquid vehicles for parenteral administration. They may include conventional, for example oily adjuvants.

The legends of figures 1 and 2, hereabove referred to, follow :

For figure 1 :

10 Control of the expression of the Iut A protein and of its precursor on two CMK 603 clones containing GTI-Iut A constructs.

PAGE-SDS pattern of total proteins of the clones:

- 11
1. E. coli CMK 603 GTI-001
 3. E. coli CMK 603 GTI-Iut A (strain 16003)
 4. E. coli CMK 603 GTI-Iut A (strain 15972)
 2. Molecular weight references.

For figure 2 :

12 Control of the expression of the Fep A protein and of its precursor.

PAGE-SDS pattern of total proteins of the clones.

- 25
1. E. coli CMK 603 GTI 001
 2. E. coli CMK 603 GTI-Fep A.

TABLE I

	PATHOGENIC STRAINS	SEROTYPE	ORIGINAL ANIMAL	REFERENCE
8	<u>E. coli</u> 15 022	O 78	chicken	Southern Quebec Ex.
	" 15 393	O 86	calf	"
	" 15 972	O 2	chicken	"
	" 16 000	O138	calf	"
	" 16 003	O138	calf	"
10	<u>E. coli</u> KM 576	(p ColV-K36)Man		B. GIDEGA
Host strains <u>E. coli</u> _ K12 genotype origin or reference				
	. C 500	F- <u>thr</u> , <u>thi</u> , <u>leu</u> , <u>lac Y</u> , <u>trp A</u> , <u>sup E</u>		(MAGATH et al.)
15	. HB 101	F- <u>hsc</u> (<u>trp</u> , <u>trp</u>), <u>trpA</u> , <u>ara</u> , <u>trp</u> , <u>lacY</u> , <u>gal</u> , <u>trp</u> , <u>trp</u> , <u>supE</u>		MAGATH et al.
	. HB 18	F- <u>thi</u> , <u>trpG</u> , <u>leu</u> , <u>trp</u> , <u>trpA</u> , <u>trpA</u>		MAGATH et al.
20	. CMK 603	F- <u>thr</u> , <u>thi</u> , <u>leu</u> , <u>supE</u> , <u>trpA</u> , <u>trpA</u> , <u>lac Y</u> , <u>trp</u> , <u>trp</u>		INSTITUT Pasteur
	. 15525	F-		Southern Quebec Ex.
Bacteriocines-producing strains				
25	<u>E. coli</u> 1300	Colicin B producing		B. PORTALIER
	<u>Enterobacter cloacae</u> DF 13 S458	Cloacin DF 13 producing		B. GIDEGA

TABLE II

	Name	Size (bases pairs)	Characteristics	Reference or origin
	pBR 322	4363	Amp ^r Tet ^r cloning vector	P. BOLIVAR
10	PAT 153	3500	Amp ^r Tet ^r cloning vector	A. TIGGS
	PSB 113	2692	Amp ^r derived from pUC18 cloning vector	P. STRASSER Institut Pasteur Paris
15	pGT10 001	5780	Tet ^r expressing vector	P. BRUNEAU Institut Pasteur
	PABN 1	16300	Amp ^r vector p Plac + 16.3 kb Hind III frag- ment of ColV-K30 bearing operon aerobactin	BRUNEAU PASTEUR
20	PMS 101	15300	Amp ^r vector pBR 322 + 11 kb Hind III fragment of <u>E. coli</u> chromosome bearing genes <u>entA</u> , <u>leuA</u> , <u>lac</u> et <u>lacZ</u>	BRUNEAU PASTEUR

TABLE III

	Day 0	Day 14	Day 28	Day 35
10	intradermic injection	intramuscular injection	intramuscular injection	intramuscular injection
	125ug	125ug	125ug	250ug
15	Blood sampling	5 ml	Blood sampling	10 ml
	Day 42	Day 49	Day 56	
20	intramuscular injection	intramuscular injection		
	250ug	250ug		
25	Blood sampling	50 ml	Blood sampling	50 ml

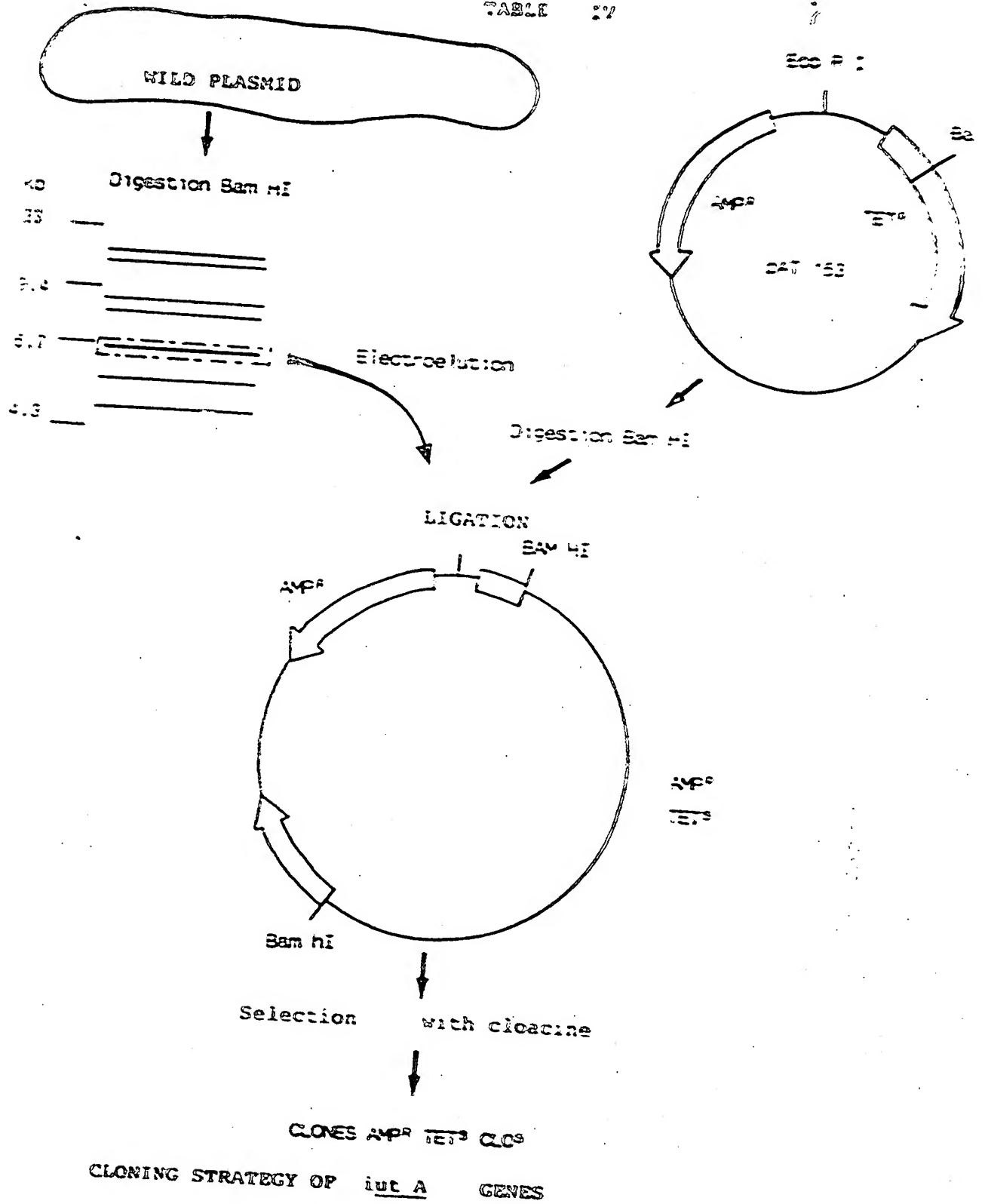
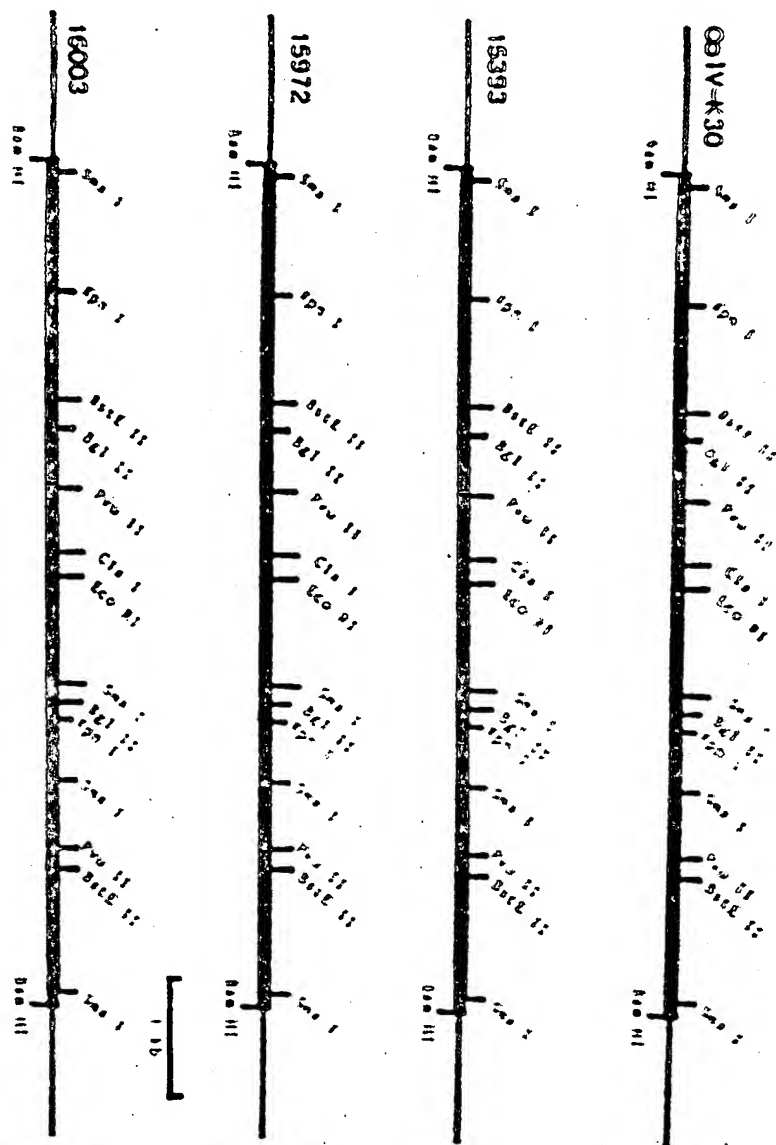
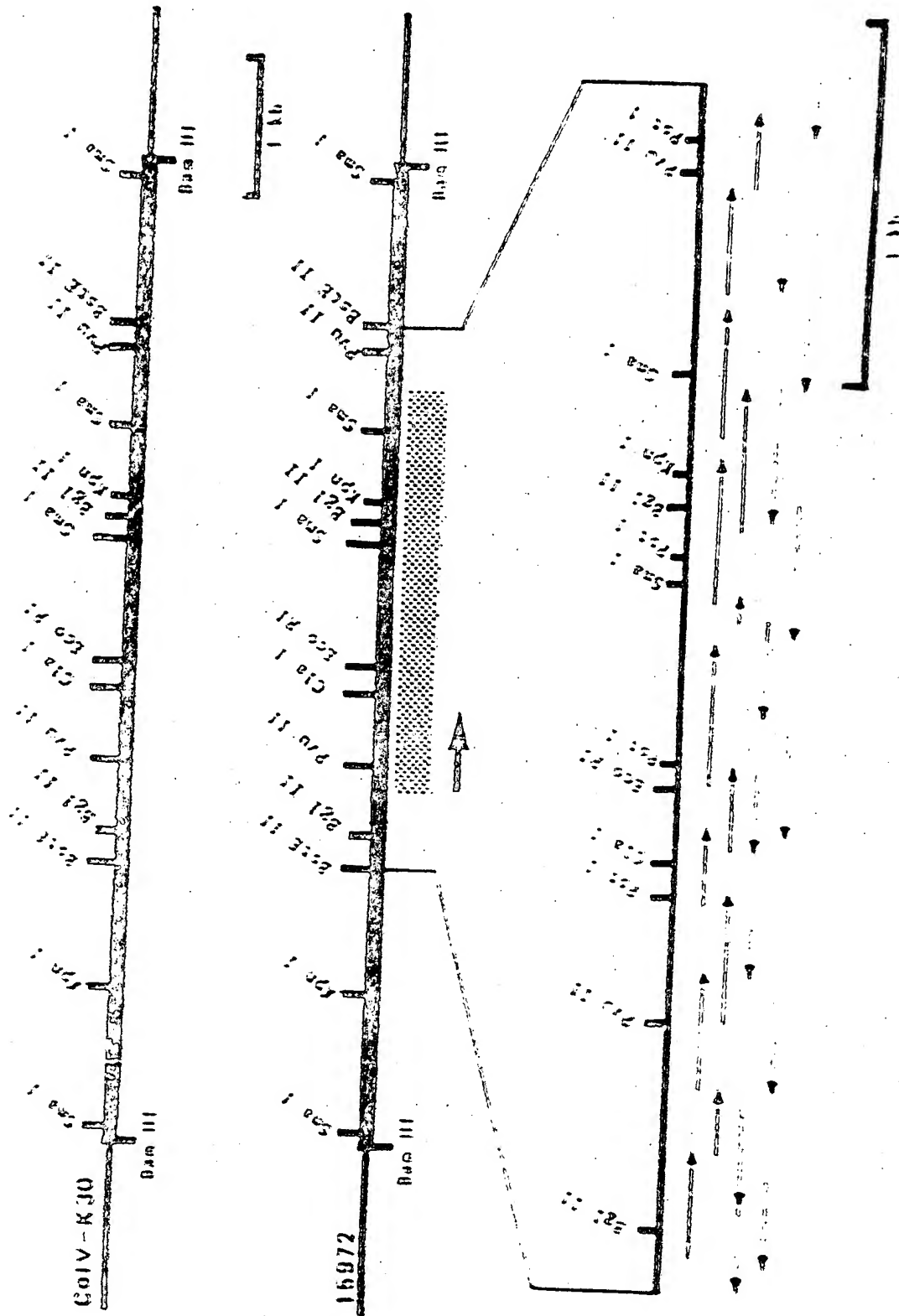


TABLE V



REGULATION MAP OF Bam HI - Bam HI fragments bearing gene loci on
 15972, 15393, 15972 and 16003



PROPOSING STRATEGY FOR GENE IN A

1
 2
 3
 4
 5
 6
 7
 8
 9
 10
 11
 12
 13
 14
 15
 16
 17
 18
 19
 20
 21
 22
 23
 24
 25
 26
 27
 28
 29
 30
 31
 32
 33
 34
 35
 36
 37
 38
 39
 40
 41
 42
 43
 44
 45
 46
 47
 48
 49
 50
 51
 52
 53
 54
 55
 56
 57
 58
 59
 60
 61
 62
 63
 64
 65
 66
 67
 68
 69
 70
 71
 72
 73
 74
 75
 76
 77
 78
 79
 80
 81
 82
 83
 84
 85
 86
 87
 88
 89
 90
 91
 92
 93
 94
 95
 96
 97
 98
 99
 100

TABLE VII: continued

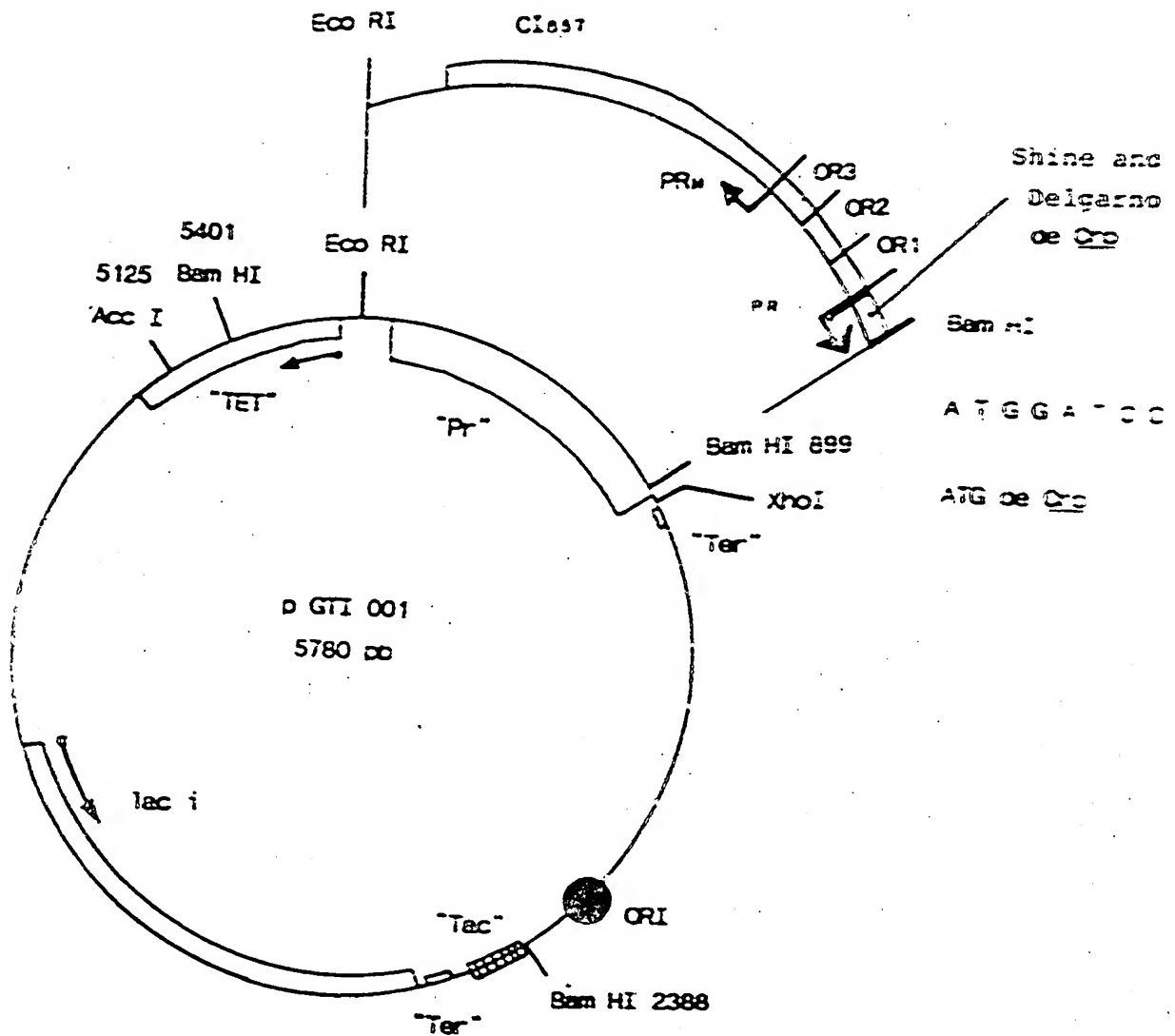
[illegible]

[illegible]

TABLE VIII

	111
ColV-K30	GTG ATC TCG GGT GCG
5393	_____
5972	-----C-----
6003	_____
	148
ColV-K30	GAC GCA TCG TCG GCG CTC AGT CGA TTA CGA GAC TCG
5393	-----C A T CCG GCG TCG CTC GAT TAC GAC AAC TCG
5972	-----C A T CCG GCG GCG TCG CTC GAT TAC GAC AAC TCG
6003	-----C A T CCG GCG GCG TCG CTC GAT TAC GAC AAC TCG
	814
ColV-K30	AAC GAA CCG CAG CAG
5393	-----GC-----
5972	-----GC-----
6003	-----GC-----
	1421
ColV-K30	GAA AAC CTC TCG GAC
5393	-----T-----
5972	-----T-----
6003	-----T-----
	2323
ColV-K30	GCG CGA CCG TCG CTC TGA Gly Pro Pro Leu Val END
5393	GCG CGA ACC TCG CTC AAC TAC TCG CTC TCG TGA
5972	-----GA ACC TCG CTC AAC TAC TCG CTC TCG TGA
6003	-----GA ACC TCG CTC AAC TAC TCG CTC TCG TGA Gly Arg Thr Pro Gly Leu Asn Val Ser Val Leu Pro END

Differences between the sequence of the three clones int A
and the sequence of gene int A DE p ColV-K30

TABLE IX
CI 697

Creation of ATG

Digestion BamHI ... ATGGATCC..

TACCTAGG..

Digestion Mung Bean 5'... ATG 3' >>> 5' ATG 3'

3'... TACCTAG 5' >>> 3' TAC 5'

PHYSICAL AND GENETIC MAP of p GTI 001

TABLE X

ACC :

ATG ATG ATA AGC AAA AAG TAT ACG CTT

MET MET ILE SER LYS LYS TRP TRP LEU

1) 5' ATGATAAGCAAAAAGT 3'

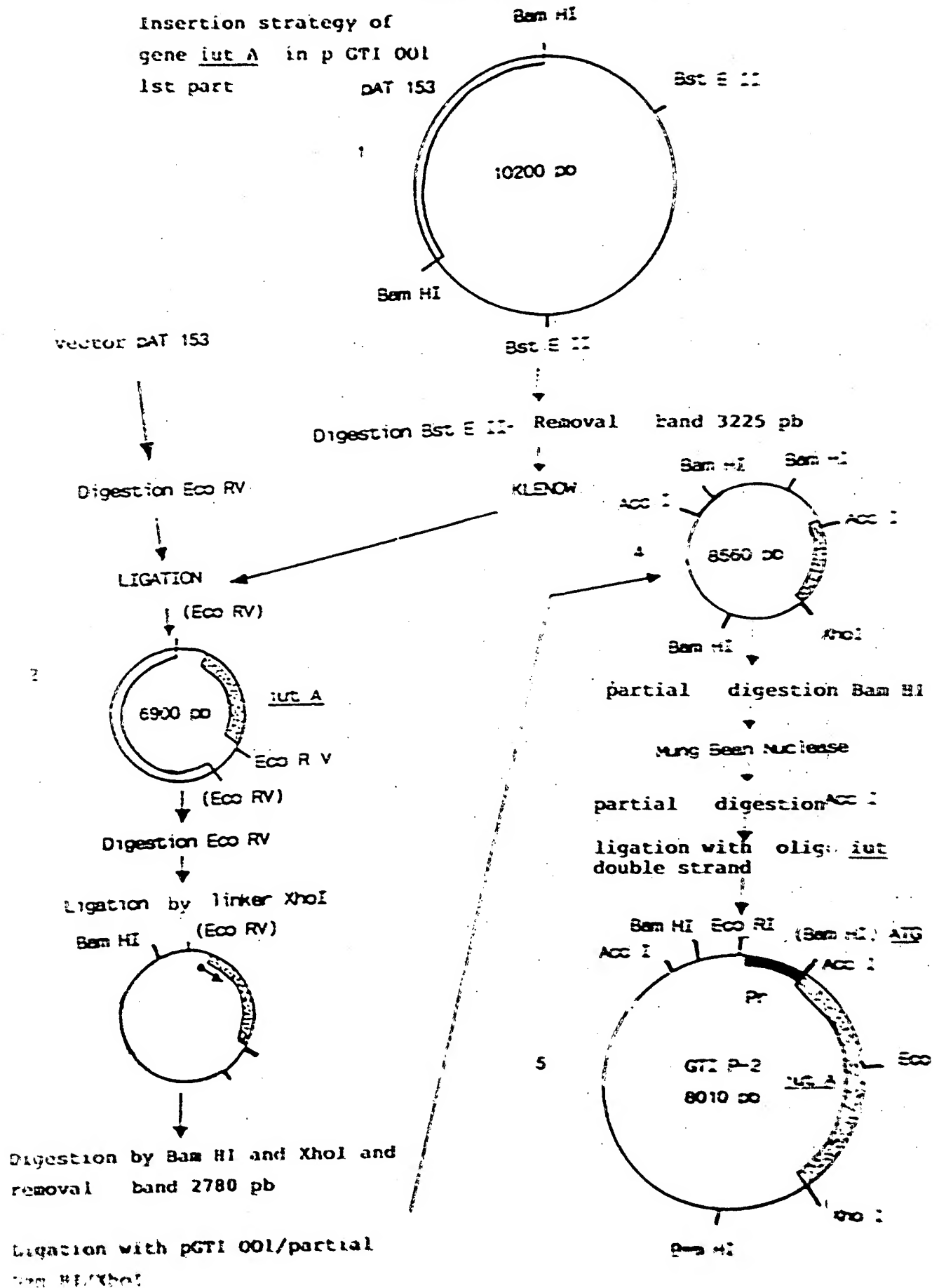
2) 3' TACTATTTCGTTTTTCATA 5'

5' ATGATAAGCAAAAAGT 3' oligo "out"
 3' TACTATTTCGTTTTTCATA 5'

Start of gene iut A and Sequence of double strand
 oligonucleotide used for the phasing of gene iut A in p GTI 00

TABLE X:

Insertion strategy of
gene lut A in p GTI 001
1st part



Bar HI EOC RI

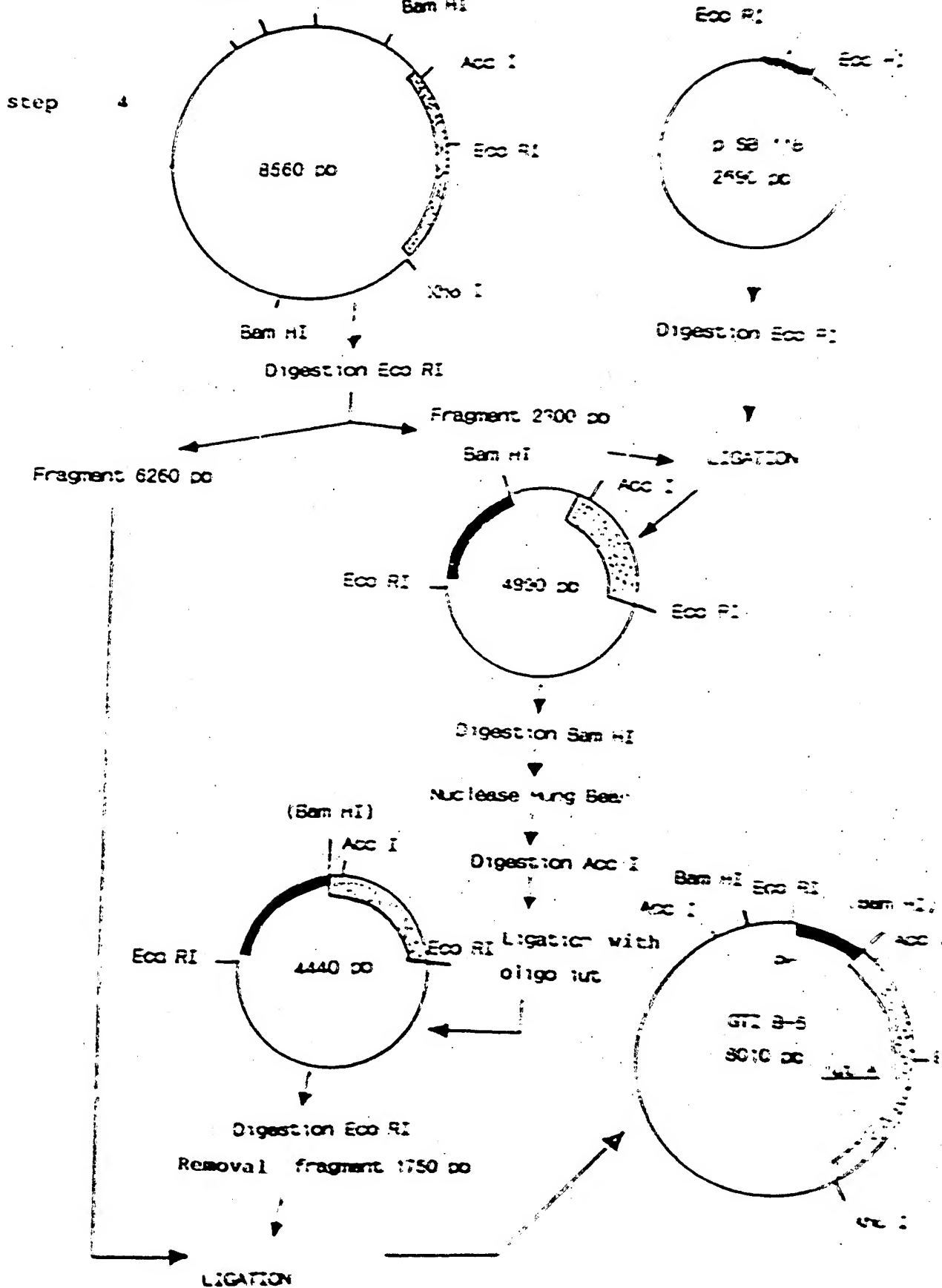
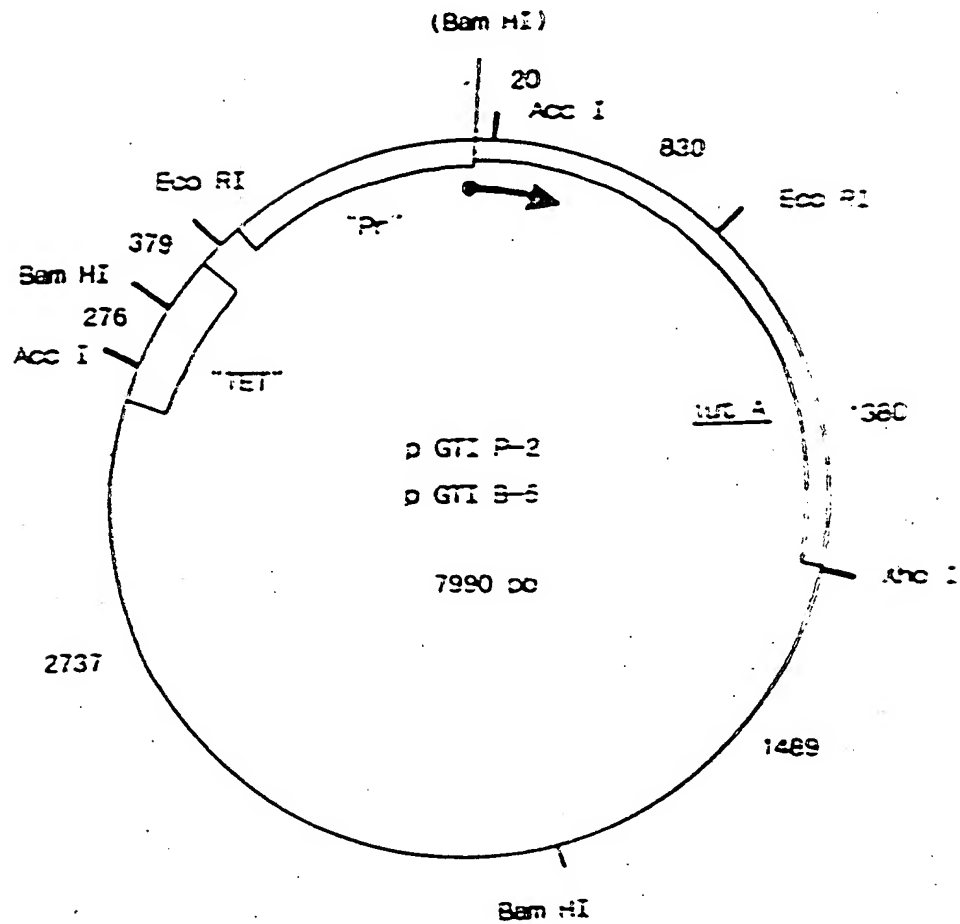
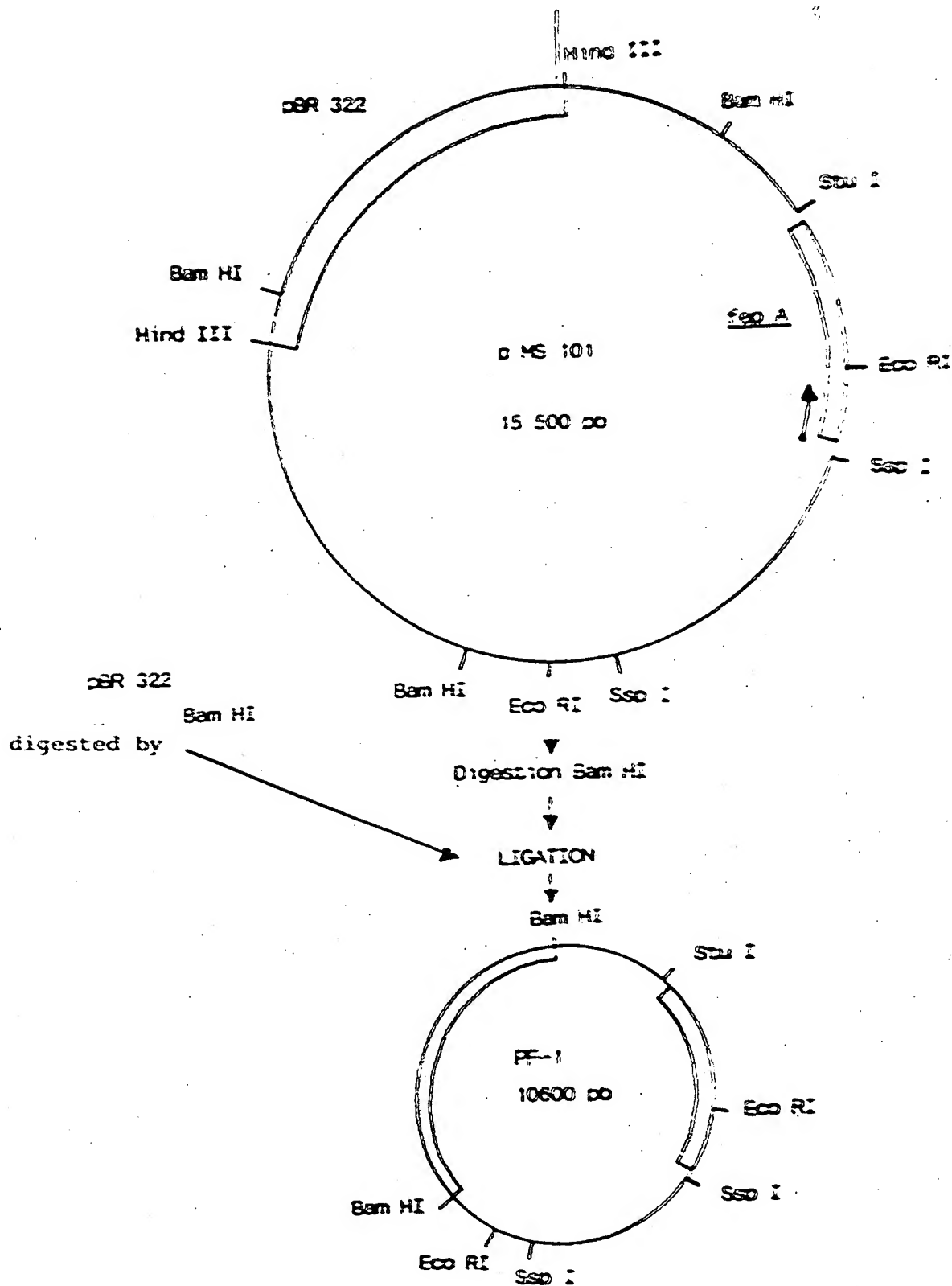


TABLE XIII

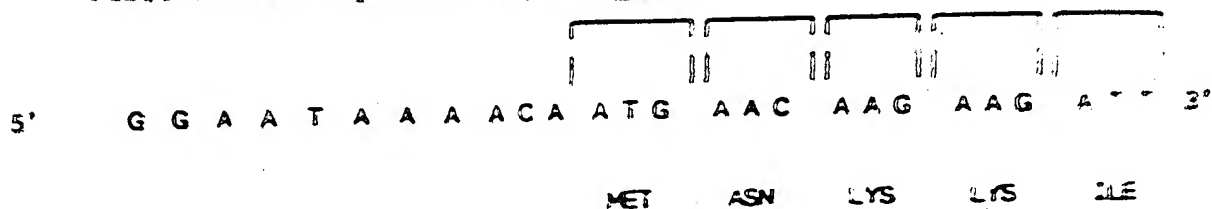


RESTRICTION MAP OF PLASMID S GTI P-2 and GTI B-5



RESTRICTION MAP OF p MS 101 and CONSTRUCTION OF p F-1

TABLE XV

start of the sequence of gene fep A

sequence to be obtained after mutagenesis :

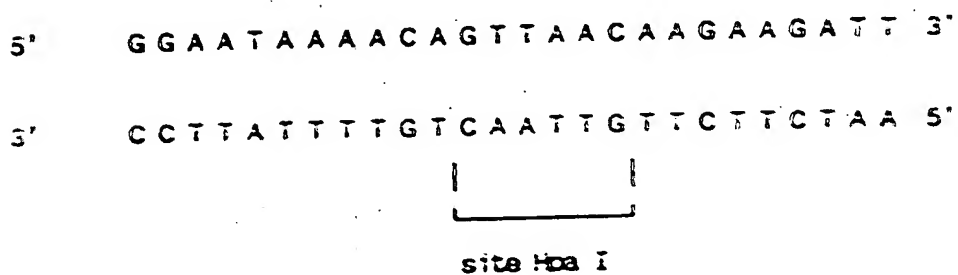
oligonucleotide (27 mer)
usedSEQUENCE OF SYNTHETIC OLIGONUCLEOTIDE USED FOR THE
MUTAGENESIS OF fep A

TABLE XVI
INSERTION STRATEGY OF GENE FEP A
IN p GT1 001

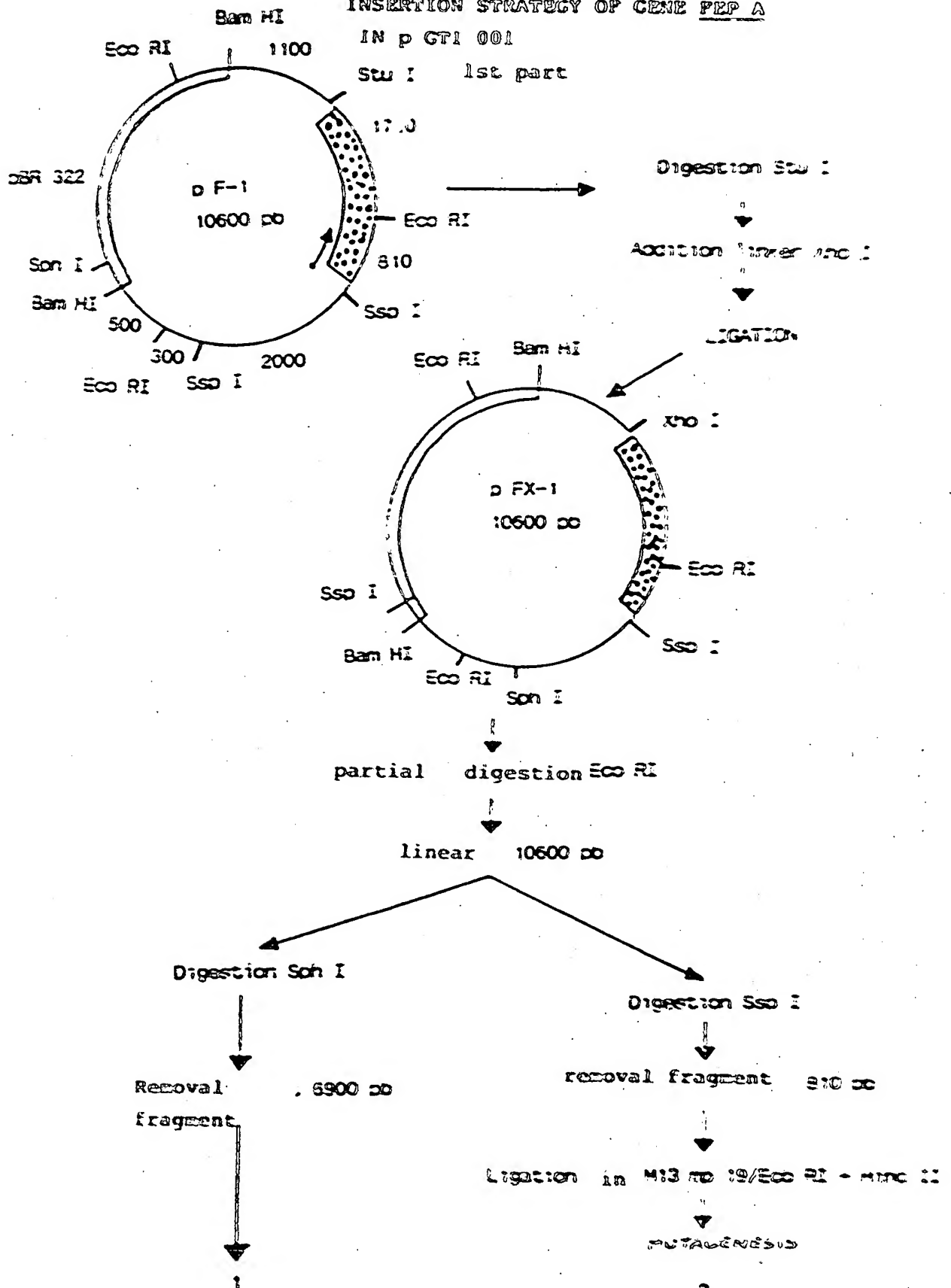
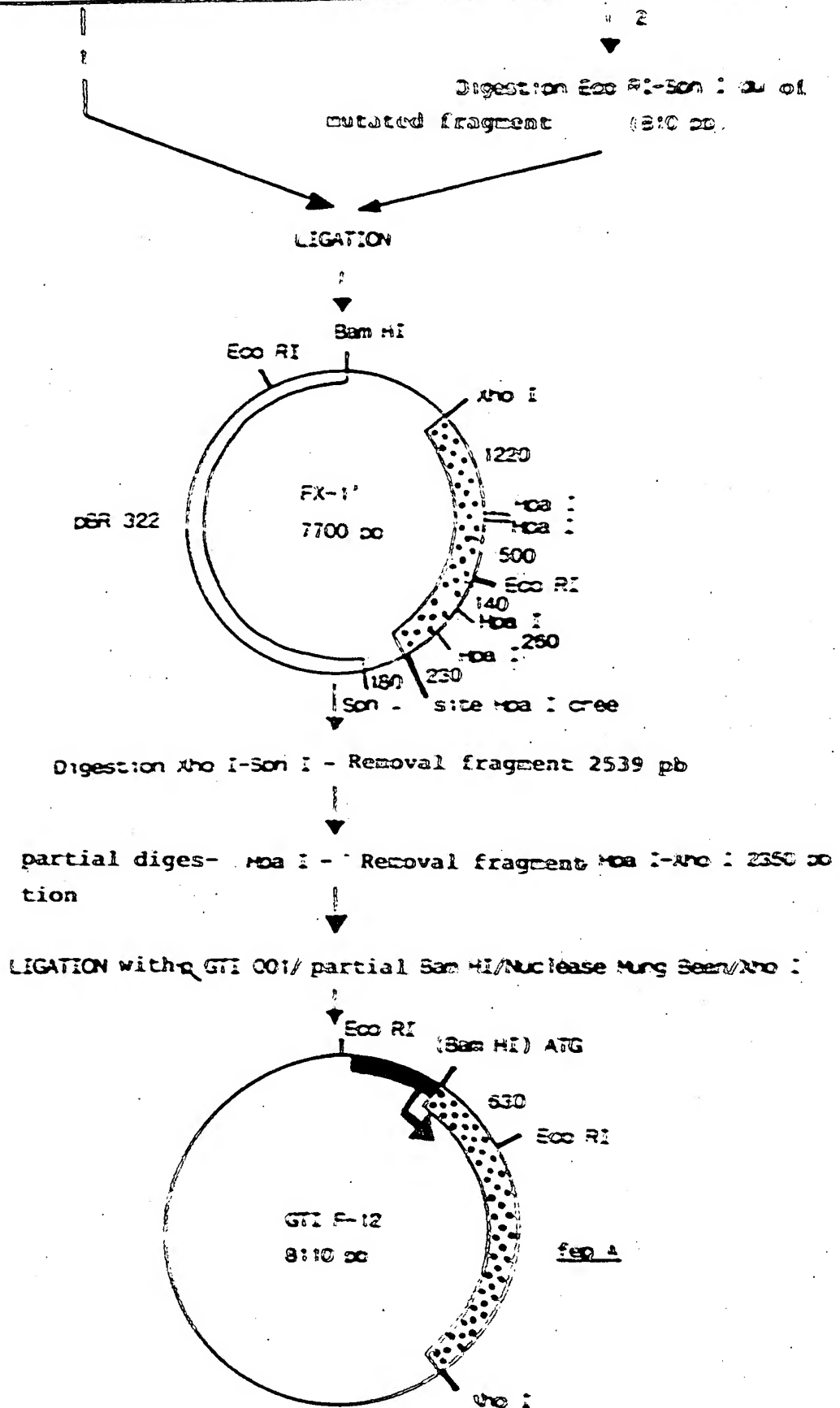


TABLE XVII



BIBLIOGRAPHY

- (1) BINDEREF A., BRAUN V. et HANTKE, J. *Bacteriol* 150, 1472-1475 (1982)
- (2) BINDEREF A. et NEILANDS J.E. *Bacteriol* 150, 1111-1113 (1982)
- (3) BERNSTEIN H.C. et SOLY J., *Nucleic Acids Res* 7, 512-522 (1979)
- (4) BOLIN C.A. et JENSEN A.B., *Infect* 15, 1242 (1987)
- (5) BYERS B.R., *Trans. Transplant* 15, 11-16, "Iron transport in microbes, plants and animals", G. WINKELMAN, D. VAN DER MEY et J.B. NEILANDS, VCH Publishers Weinheim, FRG.
- (6) CODERRE P. et ZARNHART C.F., "Characterization of a plasmid carrying the *Escherichia coli* H₂ ant. 2, *ant* 2, *ant* 3 et *ant* F⁺ genes FEMS Microbiol. Letters 25, 111-115 (1984)
- (7) COULTON J.W., *Biochim. Biophys. Acta* 717, 15-16 (1982)
- (8) DE GRAAF F.H., TIEBE G.A., WENDELAAR BONGA, J. et STOUTHAMER A.H., *J. Bacteriol* 95, 631-640 (1984)
- (9) DE GRAAF F.H., COEDVOLD-DE GROOT et STOUTHAMER A.H., *Biochim. Biophys. Acta*, 221, 566-575 (1980)
- (10) ENGWALL E. et PERLMANN, J. *Immunol* 139, 111-121 (1972)
- (11) FLEMING T.P., NARLIK M.S., NEILANDS J.E. et INTOSH M.A., *Gene*, 34, 47-54 (1985)
- (12) FISS E.H., STANLEY-SAMUELSON P. et NEILANDS J.E., *Biochemistry*, 21, 4517-4522 (1982)
- (13) GRIFFITHS E., "Iron and infection - molecular physiological and chemical aspects", J.C. STILES et E. GRIFFITH, JOHN WILEY & SONS LTD, CHICHESTER ENGLAND
- (14) HERRERO M., DE LORENZO V. et NEILANDS J.E., *Bacteriol* 170, 56-64 (1988)
- (15) HOLLIFIELD M.E. et NEILANDS, *Biochemistry* 17, 1922-1928 (1978)
- (16) KADO C.I. et JIN S.T., *J. Bacteriol* 154, 111-116 (1978)

- (17) KRONE W.J.A., GULESCA B., STEGENHUIS F. et DE GRAAF F.H., J. Bacteriol. 133, 716-721 (1983).
- (18) KRONE W.J.A., STEGENHUIS F., KONINGSSTEIN D., VAN DROFFEN C., ROSENDAL B., DE GRAAF F.H. et GULESCA B. FEMS Microbiol. 25, 153-161 (1989).
- (19) LAIRD A. J. et YOUNG I. G., Gene 11, 353-366 (1981).
- (20) LOWRY O.H., ROSEBROUGH N.J., FARRA L.A. et RANDALL R.D., J. Biol. Chem. 193, 265-275 (1951).
- (21) LUSTENGERMANN B., WELTERS J., PETERS R. VAN DER WOUF F. et VAN ALPHEN L., FEBS Lett. 52, 254-258 (1976).
- (22) LUNDIGRAN M.C. et HADNER R.C., J. Biol. Chem. 241, 10797-10801 (1966).
- (23) MANIATIS T., FRITSCH E.F. et SAMBROOK J. (1982) Molecular cloning : an laboratory manual Cold Spring Harbor Laboratory, Cold Spring Harbor NY.
- (24) NAKAMAYE K. et ECKSTEIN, Nucl. Acids Res 14, 9679-9698 (1986).
- (25) OHTSUBO H. et OHTSUBO E., Proc. Natl. Acad. Sci. USA 75, 615-619 (1978).
- (26) ROGERS H.C., SYNGE C. et WOODS V.E., Antibacterial effect of scandium and indium complexes of enantiomers of Ribostamycin *Streptococcus pneumoniae*, Antimicrob. Agents and Chemotherapy 19, 61-63 (1986).
- (27) ROGERS H.C., "Iron transport in microbes, plants and animals" G. WINKELMAN, B. VAN DER HELM, et C.B. NIELANDS, p. 221-233 (1987)-VCH Publishers, Weinheim FRG.
- (28) SANGER F., NICKLEN S. et COULSON A.R., Proc. Natl. Acad. Sci. USA 74, 5463-5467 (1977).
- (29) SCHWARTMAN C.A., J. Bacteriol. 108, 545-550 (1971).
- (30) SIMON E.H. et TESSMAN I., Proc. Natl. Acad. Sci. USA 50, 526-532 (1963).
- (31) SOUTHERN E., J. Mol. Biol. 98, 503-532 (1975).
- (32) TAYLOR C.W., SCHMIDT W., CROSSICK B., DEVEREAUX A. et ECKSTEIN F., Nucl. Acids Res 13, 8743-8750 (1985).
- (33) TAYLOR C.W., OTT C. et ECKSTEIN F., Nucl. Acids Res. 13, 8751-8755 (1985).

- (14) TOUDIN H., STAEHELIN T. et CORBON J., Proc. Natl. Acad. Sci. USA 76, 4350-4354 (1979)
- (15) VAN TIEL-MENKVELD G. J., CUDEGA B., KEMPERE D. et DE GRAAF F. K., FEMS, Microbiol. Lett. 12, 373-380 (1981)
- 5 (16) VAN TIEL-MENKVELD G. J., VELTHAMPT E. et DE GRAAF F. K., J. Bacteriol. 146, 61-68 (1981)
- (17) WILLIAMS F. H., Infect. Immun. 26, 925-932 (1979)
- (18) VANISCH-PERROX C., VIEIRA J. et HESSING J., Gent 33, 103-119 (1985)

CLAIMS

1. A process for growing bacteria expressing
outer membrane proteins regulated by iron, of
5 which some are siderophores or transferrin receptors,
which may be used in a vaccine preparation, characterized
in that said bacteria are grown in a medium
in which the iron content is reduced, with the
help of a strong iron chelating protein, such
10 as a lactoferrin, to a level allowing one to
obtain an increased expression of said receptor
proteins which is enough to induce, when said bacteria
are used in a vaccine, the generation of antibodies
preventing the specific recognition of siderophores
15 by their receptors.

2. A process according to claim 1,
characterized in that the bacteria belong to
the group made up by enterobacteriae belonging
to the families Escherichia coli, Klebsiella,
20 Salmonella typhimurium, Shigella.

3. A process according to claim 2,
characterized in that the siderophores which are
excreted by bacteria, are aerobactin and/or entero-
bactin siderophores.

4. A process according to claim 3,
characterized in that the expressed proteins are
Iut A and/or Fep A proteins.

5. An anti-septicemic bacteria vaccine
characterized in that it includes, as an active
10 principle, antigens comprising whole bacteria
is obtained by culture in a medium wherein the
iron content is reduced to a level allowing one
to obtain an increased expression of outer membrane

proteins regulated by iron, and of which some are siderophores or transferrin receptors, or fragments of these bacteria, or transferrins or siderophore receptors proteins, and notably proteins Iut A and Fe A, extracted from these bacteria.

6. A vaccine according to claim 5, characterized in that said bacteria are bacteria obtained by the process according to any of claims 1 - 4.

7. Bacteria expressing in increased amounts at least one outer membrane protein, regulated by iron and forming a transferrin or siderophore receptor, characterized in that they include recombinant expression vectors expressing said protein(s).

8. Bacteria according to claim 7, characterized in that the genes expressed in said vectors belong to the expression system of the outer membrane proteins regulated by iron inside the group consisting of enterobacteria of the families E. coli, Klebsiella, Salmonella typhimurium, Shigella.

9. Bacteria according to claim 8, characterized in that said proteins belong to the systems of aerobactin and/or enterobactin siderophores.

10. Bacteria according to claim 9, characterized in that the synthesized proteins are proteins Iut A and/or Pep A and/or their precursors.

11. Bacteria according to claim 10, characterized in that protein Iut A is obtained by a process wherein notably one isolates the plasmid part of an E. coli strain or of any other enterobacterium bearing the aerobactin operon,

one separates from the plasmid part a fragment
 bearing gene lut A, one ligates said fragment
 with a cloning vector, one inserts ^{the cloned} gene lut A
 in an expression vector, and one has protein lut
 5 A expressed by growing the clones.

12. Bacteria according to claim 11,
 characterized in that the expression vector used
 to express protein lut A has its replication origin
 located under the control of the tac promoter
 10 and that the gene to express is under control
 of the strong "Pr" promoter whose repressor is
 thermosensitive.

13. Bacteria according to 10, characterized
 in that protein Pep A is obtained by cloning gene
 15 fep A in the expression vector defined in claim
 11 or 12.

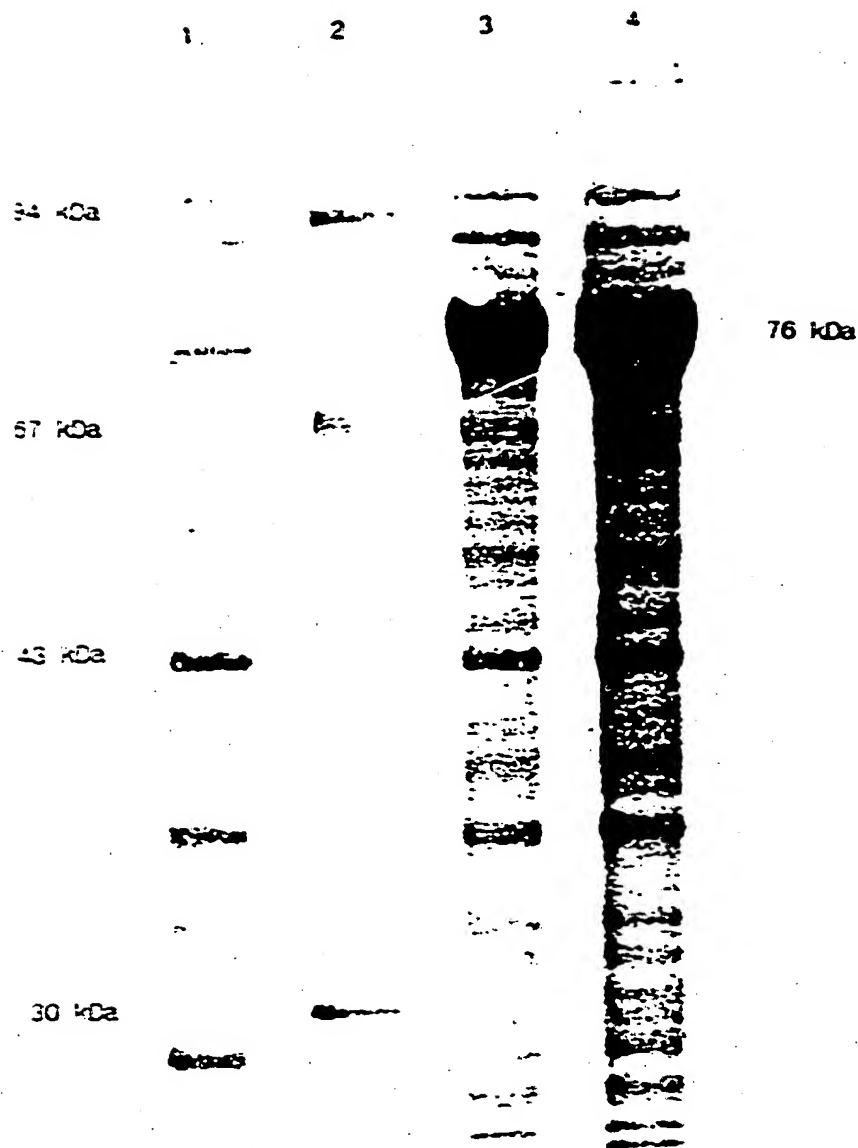
14. A process for synthesis of IROMPs
 and notably proteins lut A and Pep A or their
 precursors prolut A and proPep A, characterized
 20 in that clones as defined in claims 11-13 are
 grown in an appropriate medium, first at a temperature
 below 32°C then at 42°C to induce the expression
 of genes lut A and fep A.

15. Anti-septicemic bacterial vaccine,
 25 characterized in that it contains as an active
 principle IROMPs, and notably lut A and/or Pep
A proteins and/or the precursors of these proteins
 as extracted from the outer membrane or the cytoplasm
 of recombinant bacteria, according to any of claims
 30 7-13.

16. A vaccine according to claim 13,
 characterized in that it contains said recombinant
 bacteria or fragments of these bacteria.

Patented
 Ottawa
 Patent Office

FIGURE 1

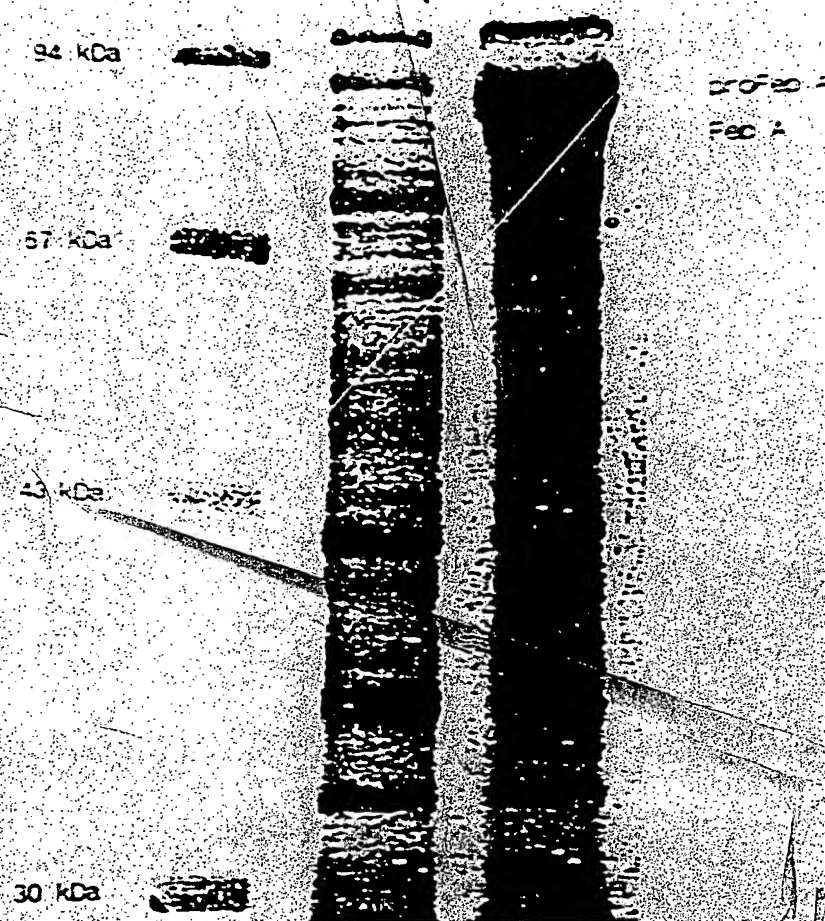


Control of the expression of protein Iut A and its precursor in two CMK 603 clones with GTI-Iut A constructions.

SDS-PAGE profiles of the total proteins in the clones :

1. E. coli CMK 603 GTI-001
3. E. coli CMK 603 GTI-Iut A (strain 16603)
4. E. coli CMK 603 GTI-Iut A (strain 15972)
2. Molecular weight standards

2



Control of the expression of protein Pep A and its precursor.

SDS-PAGE profiles of total proteins in the clones

1. E. coli CMF 603 GT1-001
2. E. coli CMF 603 GT1-Pep A